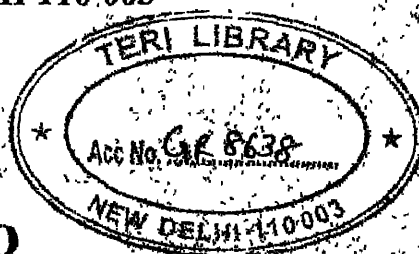


Isolation of phloem specific promoters for tissue specific expression of lectin gene in plants for insect resistance

INVESTIGATOR

MALATHI LAKSHMIKUMARAN

**BIORESOURCE AND BIOTECHNOLOGY DIVISION
THE ENERGY & RESOURCES INSTITUTE
DARBARI SEWTH BLOCK, HABITAT PLACE
LODHI ROAD, NEW DELHI 110 003**



SUBMITTED TO

INTERNATIONAL AGRICULTURAL TECHNOLOGY PROJECT

201

Format for Progress Report

1. **File No./ Sanction letter # :** 28(1)/99-NATP/CGP-1/16
DT: 31.05.2000
2. **Title of the Sub-Project:** Isolation of phloem-specific promoters for tissue specific expression of lectin gene in plants for insect resistance
3. **(a) Name of PI** Dr Malathi Lakshmikumaran
(b) Postal/Electronic address (including Email /Fax/Phone) The Energy and Resources Institute
Darbari Seth Block, Habitat Place
Lodhi Road, New Delhi – 110 003
Tel: 24682100 / 24682111
Fax. 24682144 / 24682145
E mail: malaks@teri.res.in
4. **Mode of Research** CGP
5. **Period of report** Final
6. **Date of start of the Project** 1st April' 2000
7. **Date of termination (as per sanction letter)** 31st March' 2003
8. **Objectives of the Sub-Project**
 - 1 To isolate and characterize phloem-specific promoters such as the sucrose synthase promoter from *B juncea*.
 - 2 Deletion analysis of the cloned promoter sequence to identify the core promoter sequence
 - 3 To produce transgenic *B juncea* containing snowdrop lectin gene under the control of constitutive promoter such as the 35S promoter of Cauliflower Mosaic Virus (CaMV).
 - 4 To transform *B juncea* with the snowdrop lectin gene under the control of phloem specific promoter described above.
 - 5 To test the putative transgenic plants in the laboratory and to establish tissue specific expressions of lec GNA
 - 6 To test the putative transgenics for resistance against aphids under containment.
9. **Executive summary of the work done during the year (not more than one page)**

Brassica juncea is the major oilseed *Brassica* in India, accounting for more than 80% of the land under *Brassica* cultivation. The crop *Brassica* is affected by a number of fungal infections and are also damaged by pests. The major devastation is caused by a species of the sap sucking insect, namely aphids (*Lipaphis ertsimae*). On an average,

40% loss in yield due to aphids infestation in *B. juncea* is reported. It is therefore, desirable to develop *B. juncea* plants resistant to aphids. The lectin protein obtained from snowdrop lectin (*Galanthus nivalis* agglutinin, GNA) has been shown to impart resistance against these sap sucking insects. This project therefore aims at developing the *B. juncea* lines resistant to aphids by genetically engineering the crop by introducing the lectin gene. In addition to this, we propose to isolate phloem specific promoters from *A. thaliana* and *B. juncea* and use them to drive the expression of the insecticidal lectin gene. Expression of the transgene only in the phloem tissue and not in seeds will lay to rest the fears in minds of people about issue related to the transgenic tissue.

This project has two components namely, isolation of phloem-specific promoters and genetic transformation of *B. juncea* using snow-drop lectin gene with different promoters (CaMV35s, super promoter and phloem-specific promoters). It has been reported that there are two classes of the sucrose synthase gene in *Arabidopsis* and *Brassica* species. One of them is specifically expressed in the phloem. The phloem-specific promoter from *A. thaliana* and *B. juncea* has been isolated using different methods. These include construction of genomic library, PCR walking and direct amplification from the genomic DNA. From the literature and the EMBL database we have obtained sequences of the *A. thaliana* sucrose synthase genes, which are expressed in phloem. Based on this sequence information, we have designed probes and primers to isolate this sucrose synthase promoter. The promoter has been cloned and characterized from both the species. The promoter isolated is being screened for phloem-specific nature using gus-intron as the reporter gene. Work on the transformation of *B. juncea* using the snow-drop lectin gene under the control of both 35S and superpromoter is in progress.

10 Objective-wise Achievements of the Sub-project (limit to not more than 10 pages including illustrations/photographs/tables). Every achievement should have clear quantified statements.

See Annexure - I

11 Outcome/Impact (quantified) of the achievements and the project as a whole on advancement of science/technology development/farmers.

A number of plant promoters have been identified in this project. We have cloned and characterized a phloem-specific promoter from *Brassica* species. This promoter was derived from the region upstream of the sucrose synthase gene. This promoter is useful for expression of genes in the phloem of plants. We have used this promoter to drive the expression of snowdrop lectin gene in *Brassica juncea*. These will be useful for the protection of plants against sucking insects such as aphids and the gene is expressed only in the phloem as aphids are phloem feeders. This project has helped in cloning a number of promoters and identifying motifs present in the promoter which are responsible for organ specific expression in plants.

12 Foreign / National Training's obtained by the PI & the CCPI

None

- | | |
|--|---|
| 13. Training arranged, if any | None |
| 14 List the Manuals / Instructional Material / CDs / extension material developed | None |
| 15 Infra-Structural development
(List only equipments and works costing more than Rs 5.00 lakh each) | Genescan and Genotyper for the use with automated sequencer |
| 16. Improvement in audio-visual system (if any) | None |
| 17. List the papers published based on work under NATP in refereed journals | None |
| 18 Honors and awards received based on work under NATP | None |
| 19. Technologies developed (if any) | None |
| 20. Patents applied for/obtained | None |
| 21 Your suggestion regarding what has to be done in future as a continuation of your project or as a new project | |
- Promoters are useful for expression of genes in plants. It is important to identify, clone and characterize organ specific promoters in plant species. We have developed a PCR walking strategy to isolate promoters upstream of many genes. We have used phloem- species promoters to drive the expression of snow drop lectin gene in *Brassica juncea*. We have produced few plants using *Agrobacterium* mediated transformation. We need to produce many more number of plants to obtain a transformed plant which shows protection against insects and is also high yielding.


Signature PI


Signature Director/ Director of Res.

Introduction

Brassica species represent the most important vegetable, oilseeds and technical crops. In India, Brassica ranks second in acreage superseded only by groundnut among the major oilseed crops. The two species namely *Brassica juncea* and *B. campestris* occupy the maximum area under cultivation in India. Mustard seeds are widely used as condiments in food and as source of an array of food additives. Production of mustard in India has shown a steady increase from 3.46 million tons (1987-88) to 5.7 million tons (1995-96) in response to increasing demands. Most of the increase in the production has taken place due to an expansion in the area under cultivation from 2.1 to 6.3 million hectares. Despite a large number of efforts involving traditional breeding programs, the average yield under Indian cultivation practices remains well below the world average mainly because of its susceptibility to different biotic and abiotic stress. Hence, it is imperative that future crop improvement programs need to employ new biotechnological approaches. Increasing crop productivity through genetic transformation is one of the major areas of research these days. Even though majority of the worlds major crop plants can now be transformed, what is lacking are the functional genes and novel promoters for transfer into host plant.

All over the world Brassica crops are damaged by different diseases which include white rust (*Candida albugo*), alternaria blight, powdery mildew etc. *Brassica juncea* (mustard) is also known to be infested by a large number of insects and pests. The major devastation is caused by a species of aphid viz. *Lipaphis estsimae* (Indian mustard aphid) (Chopra and Prakash, 1996). Both mymphs and adults damage the plants by sucking sap from leaves buds and stem through their prominent stylets. Due to very high pest populations, the vitality of the plants is highly reduced causing them to become pale with curled leaf tops. In addition to the direct losses caused by aphid pathogenecity, they also act as vectors for transmission of plant viruses. On an average an alarming 40% loss in total yield may be caused. Hence, it is desirable to produce insect resistance Brassica plants. Attempts to obtain insect resistant transgenic crop plants have been made through the introduction of genes encoding Bt endotoxins, protease inhibitors or lectins. Of these, former two are most effective against insect species belonging to the Lepidptera and Coleoptera orders. Whereas, lectins have been shown to impart resistance against sap sucking insects belonging to the order hemiptera.

Lectins are carbohydrate-binding proteins found in plant tissues. Lectins are found in majority of the plant tissues with abundance in the seeds and storage organs of some plant species. The seeds of certain legumes are particularly rich source of lectins, for example the

Phaseolus vulgaris lectin may be present at levels of upto 3%. It is assumed that lectins play fundamental biological roles in the plant defense mechanism (Goswami et al., 1998). These have been shown to be toxic to insects as they bind to the midgut epithelial cells. Toxicity of this type of proteins in mammals and birds have been well documented and detailed studies on their mechanism of toxicity have been carried out (Liener, 1980; de Oliveira, 1986).

The first evidence of proteins being effective against sap sucking insects was obtained from snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) (Janzen et. Al., 1976). This lectin exhibited toxicity against brown planthopper (*Nilaparvata lugens*). The gene encoding snowdrop lectin has since been isolated and successfully transferred into tobacco, sweet potato and potato (Newell et. Al. 1995; Gatehouse et. Al., 1997). Preliminary results have indicated that these transgenic plants show significant reduction in insect biomass and leaf damage. Further investigations also revealed that GNA besides being toxic to certain homopterans such as planthopper also affects the survival and development of insects belonging to other classes. In addition to this, the use of phloem specific promoter to express the insecticidal lectin protein will be especially useful for conferring resistance to the sap-sucking aphids. Expression of the transgene only in the phloem tissue and not in the seed will lay to rest the fear in the minds of people about issue related to transgenic food.

Overall progress since the implementation of the project

This project involves two broad approaches:

- I. Isolation of phloem-specific promoters**
- II. Genetic transformation of *Brassica juncea***

I Isolation of Phloem-specific Promoters

Materials and methods

Modified CTAB procedure (Doyle and Doyle, 1990) for Genomic DNA extraction

Lyophilized leaf material (500 mg) was ground to a fine powder using mortar and pestle. Silica gel was added as an abrasive. 15 ml of prewarmed (65 °C) CTAB extraction buffer was added to the pulverized leaf material, mixed thoroughly, and the slurry was transferred to capped polypropylene tube. The samples were incubated at 65 °C for 30 minutes with intermittent shaking every 10 minutes. An equal volume of chloroform: isoamyl alcohol (24:1) was added to the DNA samples and mixed thoroughly to ensure emulsification of the phases. The samples were centrifuged for 20 minutes (5000 X g, 25 °C) and the upper aqueous phase was transferred to a fresh clean tube. RNase A (10 mg/ml), which was free of DNase, was added to a final concentration of 100 µg/ml and the samples were incubated at 37 °C for 30 minutes. The samples were re-extracted with fresh chloroform: isoamyl alcohol. The final aqueous phase was transferred to 'Corex' glass tubes with the help of a large bore pipette.

Ice-cold isopropanol (0.6 volumes) was added to precipitate the DNA. The precipitated DNA was either spooled with help of a bent hook or pelleted by centrifugation (5000 X g, 4 °C). In all events, where the DNA precipitate was not observed after centrifugation, the samples were incubated at -20 °C for 30 minutes to overnight and pelleted at higher centrifugal force (8000 X g, 4 °C). Residual CTAB was removed by adding 20 ml of washing solution (10 mM ammonium acetate in 70% Ethanol) and gently agitating the pellet for a few minutes. The pellet was collected by centrifugation (10 minutes, 5000 X g, 4 °C) and allowed to dry. The pellet was dissolved in an appropriate volume (1 ml) of double distilled water (ddw) or TE buffer.

Plasmid miniprep by alkaline lysis method

Plasmid DNA was extracted as per the protocol given by (Birnboim 1983). Bacterial cells containing recombinant plasmids were grown overnight at 37 °C in a 2 ml culture. The cells were pelleted down for 5 minutes and the supernatant was discarded. The pellet was resuspended by vortexing in 100 µl of ice cold "Solution I" and was incubated for 5 minutes at room temperature. "Solution II" (200 µl) was added and the contents were mixed by

inversion. This was followed by incubation on ice for 5 minutes. Finally, 150 µl of ice cold "Solution III" was added and mixed by inversion followed by further incubation on ice for 5 minutes. The suspension was centrifuged for 10,000 X g for 15 minutes and the supernatant was collected. RNase A was added to the supernatant to a final concentration of 20 µg/ml and was incubated at 37 °C for 30 minutes. The mixture was extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1, v/v) and centrifuged for 15 minutes to separate the phases. The aqueous phase was transferred to a fresh tube and re-extracted with equal volume of chloroform: isoamyl alcohol (24:1). To this, 1/10th volume of sodium acetate (pH 4.8) followed by 2.5 volumes of absolute alcohol was added. DNA was precipitated at -20 °C for 30 minutes and then pelleted by centrifugation. The pellet was either dissolved in 20 µl of ddw or further purified by PEG method. For PEG purification, the pellet was dissolved in 16 µl ddw. Following this, 4 µl of 4 M NaCl was added. After thoroughly mixing the contents, 20 µl of 13% PEG (w/v) was added and the samples incubated on ice for 20 minutes. The samples were centrifuged for 10 minutes to remove the supernatant and the pellet washed with 1ml pre-chilled 70% ethanol followed by absolute alcohol. The pellet was air dried and dissolved in desired amount of double distilled water or TE buffer.

Screening of the positive recombinants by colony PCR

PCR amplification of the cloned inserts was carried out on colonies (colony PCR). A part of colony was picked up with sterile tooth pick and re-suspended in the PCR reaction mix.

The reaction was assembled as follows:

10X PCR Buffer	1.5 µl
25 mM MgCl ₂	1.5 µl
10 mM dNTP's	0.3 µl
Primer T7/Forward Primer (20 pmol/µl)	0.6 µl
Primer SP6/Reverse Primer (20 pmol/µl)	0.6 µl
<i>Taq</i> polymerase (5 U/µl)	0.1 µl
ddw	11.0 µl
Total Volume	15.0 µl

STRATEGY ADOPTED FOR ISOLATION OF PROMOTER SEQUENCES FROM BRASSICA SPECIES

Isolation of high molecular weight total DNA from young leaves



Partial time course restriction of genomic DNA with *EcoRI*



Ligation of the restricted genomic DNA to Lambda *ZapII* vector (cut with *EcoRI*) and packaging *in vitro*



Plating and titre determination of the library



Screening the plaques to homogeneity for promoter and gene of sucrose synthase



Subcloning the positive recombinants into plasmid vectors



Restriction enzyme analysis



Generating *ExonucleaseIII* deletions and screening the deletion clones



Molecular characterization of the recombinant clones
Southern hybridization and sequence analysis.

Fig. 1: Flowchart showing the strategy for construction of genomic DNA library using Lambda *ZapII* vector .

The reaction mix was overlaid with a drop of mineral oil and placed in a thermal cycler. An initial denaturing step was given at 94 °C for 1 minute followed by:

1 cycle of: 4 minutes at 92 °C 1minutes at 55 °C 2 minutes at 72 °C	30 cycles of: 1 minutes at 92 °C 1minutes at 50 °C 2 minutes at 72 °C	Final extension: 7 minutes at 72 °C
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The positive clones were grown in 2 ml cultures (L-Broth, Ampicillin 100 µg/ml) overnight and plasmids were isolated. The sequences of some of the universal primers utilized are as follows:

Primer	Sequence
SP6 (19mer)	5'-TATTTAGGTGACACTATAG-3'
T7 (20mer)	5'-TAATACGACTCACTATAGGG-3'
Lec F (22mer)	5' GGTGTCATCACACCATCTTGC-3'
LecR (24mer)	5' CACAAGCTTTATCTTTCCAGC-3'

I Isolation of Phloem-specific Promoters:

The rationale for isolation of phloem specific promoters is that we would like the expression of the snowdrop lectin gene in the phloem tissues. This is because aphids are phloem feeders and it is necessary for the lectin to be expressed in phloem and not in other organs. With this objective, we have initiated work to isolate the phloem-specific promoters from *Brassica* species. We are following the two strategies:

1. Construction of Genomic Library in Lambda Vectors:

Genomic DNA was isolated and partially digested with *Eco* RI and ligated into the lambda arms. The recombinant lambda was packaged using the packaging mix. The plaques were screened with a heterologous probes of sucrose synthase from *Arabidopsis thaliana*. The strategy for isolating the promoters for genomic DNA using lambda vector given in **Figure 1**.

Methodology for cloning into lambda vectors:

i) Preparation insert DNA

- 10µg of *B.juncea* cultivar Pusa Bold was digested with EcoRI in 100µl total volume.
- 5µl checked on agarose gel for digestion.
- Remaining digested DNA was extracted with phenol. chloroform followed by chloroform: iso amyl alcohol.
- DNA was precipitated by adding 1/10 volume of 3M Sodium acetate and 2 volumes of absolute ethanol.
- Dissolved DNA in 100µl of TE.

ii Sucrose Density fractionation:

- Preparation of 40% sucrose density gradient containing 25% sucrose, 1M Sodium Chloride, 20 mM Tris (pH-8.0) and 5mM EDTA.
- Digested purified DNA layered on to sucrose gradient.
- The tubes were centrifuged at 25,000 rpm in SW40 Ti rotor for 24 hrs at 20° C.
- Fractions of 0.5 ml collected and 20 µl fractionated on agarose gel.
- Fractions containing required fragment size were pooled and precipitated and dissolved in 20 µl TE . **Figure 2** shows the different size fractions of genomic DNA.

iii Ligation of fractionated DNA into lambda ZapII vector:

The schematic diagram of lambda Zap II vector is shown in Figure 3.

Lambda ZapII EcoRI(1µg/µl)	1.0 µl
10 X Ligation buffer	1.0
10 mM ATP	1.0
Insert DNA (200ng/µl)(1-5 kb)	2.0
T4 DNA ligase	1.0
Water	4.0
Total	<u>10.0 µl</u>

iv In vitro packaging of ligated DNA

- Ligated DNA 1- 4 µl was added to 15 µl of freshly freeze thawed Gigapack II Gold packaging extract(Stratagene , USA).
- Incubated at RT for 2 hrs .



Fig.2 **Fractions (1 to10) after sucrose gradient loaded on agarose gel. M is the marker.**

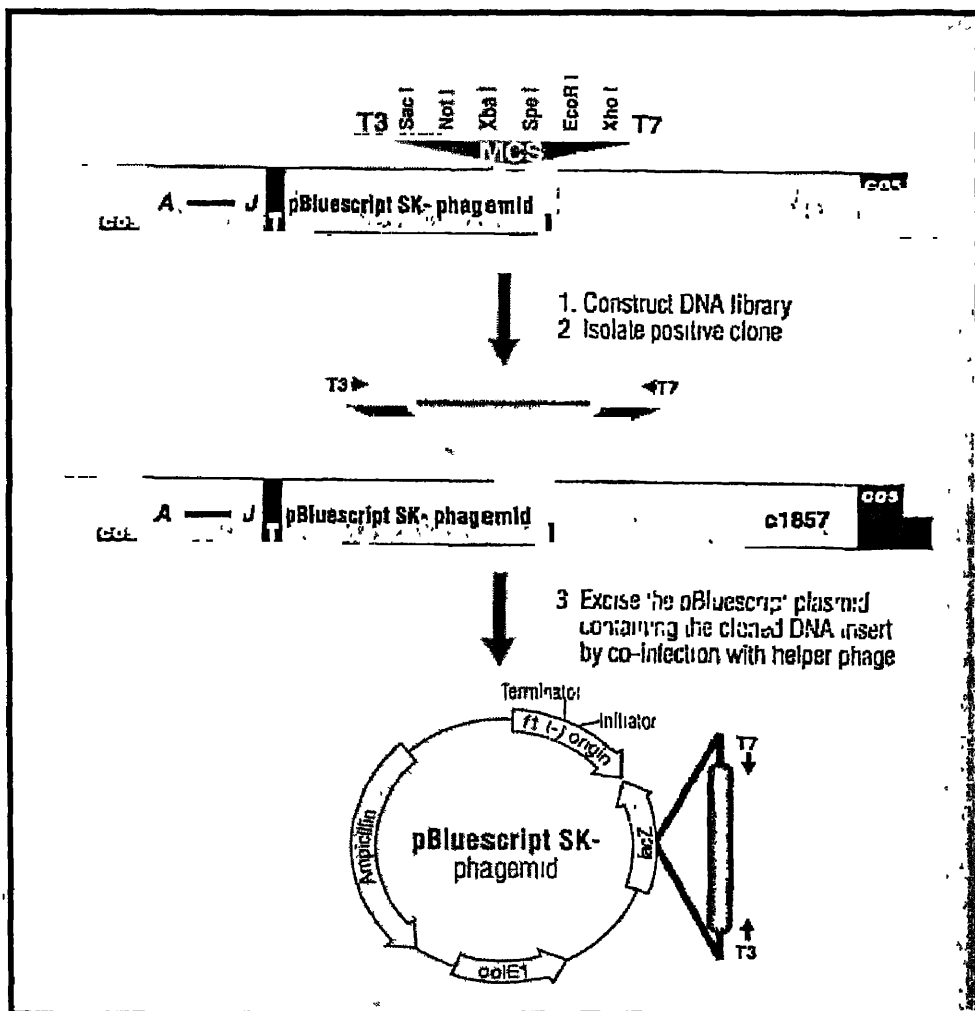


Fig. 3: Diagrammatic representation of the Lambda Zap II vector and pBluescript SK-phagemid vector showing the multiple cloning sites.

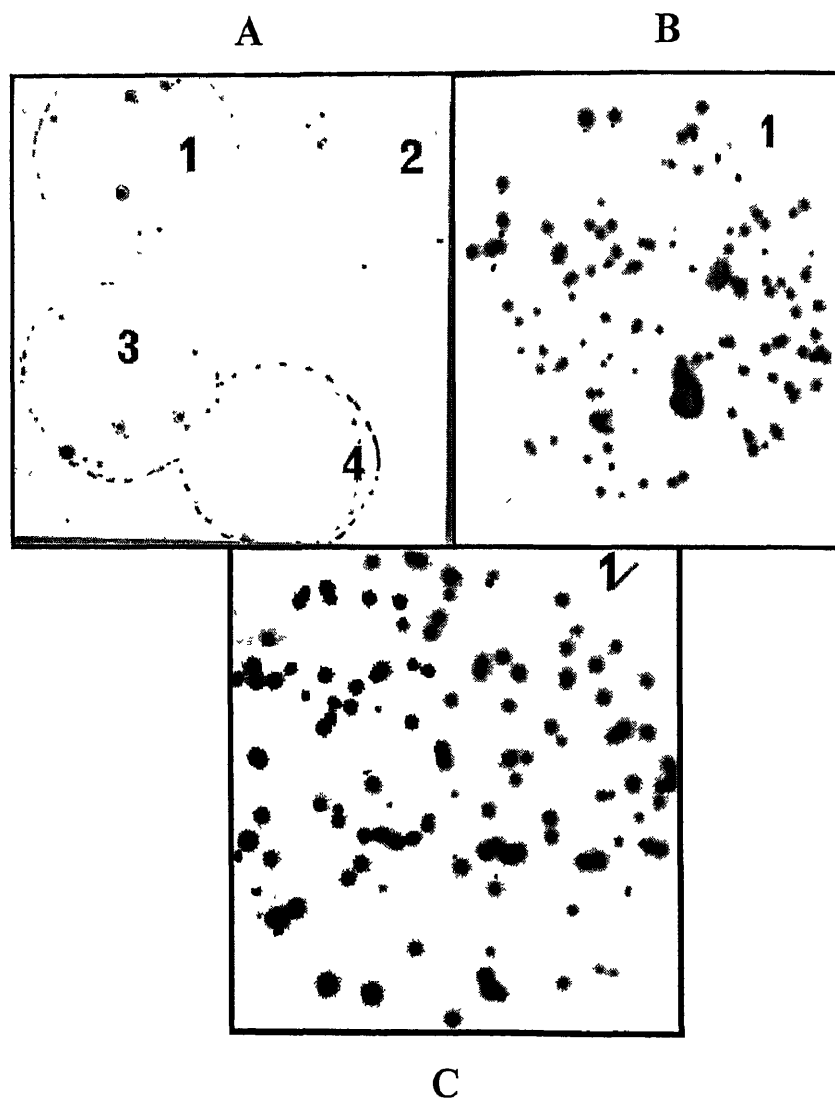


Fig. 4: Hybridization of sucrose synthase gene from *Arabidopsis* to the plaque lifts from *B.juncea* lambda library. A ,B, and C represent the plaque hybridization after primary, secondary and tertiary blotting respectively.

- 500 µl of phage dilution Buffer was added with 20 µl of chloroform and stored at 4 °C.

v Library Amplification

- 20µl packaged digested DNA was mixed with 600 µl of host cells *E.coli*; ER1648 and incubated at 37 °C for 30 minutes
- 9 ml of top agarose added to the above and poured on T B Bottom plates.
- Incubated plates for 6 – 8 hrs at 37° C
- 10 ml of phage Buffer added to each plate and kept for slow shaking at 4 °C for 3 – 4hrs.
- The top of the plate removed and centrifuged at 5000 rpm to get clear lysate.

vi Preparation of Host / plating cells

- Single colony of *E.coli* ER1648 was inoculated in 50 ml TB medium containing 0.2% maltose and 10mM Magnesium Sulphate and grown overnight at 37° C at 200 rpm
- Cells are pelleted by centrifugation at 5000 rpm for 10 minutes at 4 °C.
- The pellet suspended in 10 mM magnesium sulphate to an OD 580 of 0.5.

vii Determination of Titre

- Dilutions 10⁻¹ to 10⁻⁴ of a amplified library in 100µl of the phage buffer
- The dilutions were mixed in 200µl host cells and incubated at 37° C for 30 minutes
- 3ml top agarose was added to each dilution and poured onto T B plates.
- Plates incubated at 37° C for 6 – 8 hours

viii Plaque Blotting

- The plaques 100 – 200 per plates were lifted on Hybond N membrane.
- The membrane is then placed on Whatman sheet soaked with denaturation solution for 3 minutes.
- The membranes were finally placed on Whatman sheet saturated with neutralizing solution for 3 minutes.

ix Hybridization of Plaque lifts:

The membrane containing plaques were hybridized with hetrologous probes such as sucrose synthase from *Arabidopsis*. On primary screening only few recombinant plaques showed signal as seen in **Fig. 4A**. The positive plaques were picked and suspended in 100 µl phage buffer. These plaques were again plated and screened with the radioactive probe for secondary screening(**Fig. 4B**). The positive plaques were again picked up and suspended in

100 µl of phage buffer. An aliquote of 1 µl of the above was plated for tertiary screening and it is clear from the **figure 4C**, that all the plaques showed signal after the radiolabel probe was added. Thus we obtained recombinant plaques containing the sucrose synthase gene fragments as inserts. We could obtain 6 different recombinant plaques from the library.

II Strategy for isolation of Phloem specific promoter(Sucrose synthase) from *B.juncea*/*Arabidopsis* by PCR Walking technique:

It has been reported that there are two classes of sucrose synthase in *Arabidopsis* and *Brassica* species. One of the sucrose synthase is specifically expressed in phloem. From the literature and EMBL data, we have obtained the sequences of the *Arabidopsis thaliana* sucrose synthase genes, which expresses in the phloem. Based on the sequence we designed primers to walk upstream of the Sucrose synthase gene in both *Arabidopsis thaliana* and *Brassica* species. The principle for PCR walking is given in **Figure 5**. The PCR walking methodology is as follows:

PCR walking (Siebert et al. 1995) was applied with modifications as suggested by Devic et al. (1997) for isolating promoter sequences such as sucrose synthase.

PCR Walking Method

i. Restriction of genomic DNA

Take separate aliquots of 2.5 µg to 3 µg good quality, high molecular weight genomic DNA and digest with *Eco* RV, *Dra* I, *Pvu* II, *Sca* I and *Ssp* I which are blunt end producing enzymes. Use excess enzyme to ensure complete restriction of DNA.

Set up four separate restriction reactions each containing:

DNA	5.0 µl (500 ng/µl)
10 X buffer	10.0 µl
Enzyme (50 U)	5.0 µl (10 U/µl)
Rnase A (10mg/ml)	1.0 µl
Water	79.0 µl

	100.0 µl

Spin for few seconds.

Incubate overnight at 37°C.

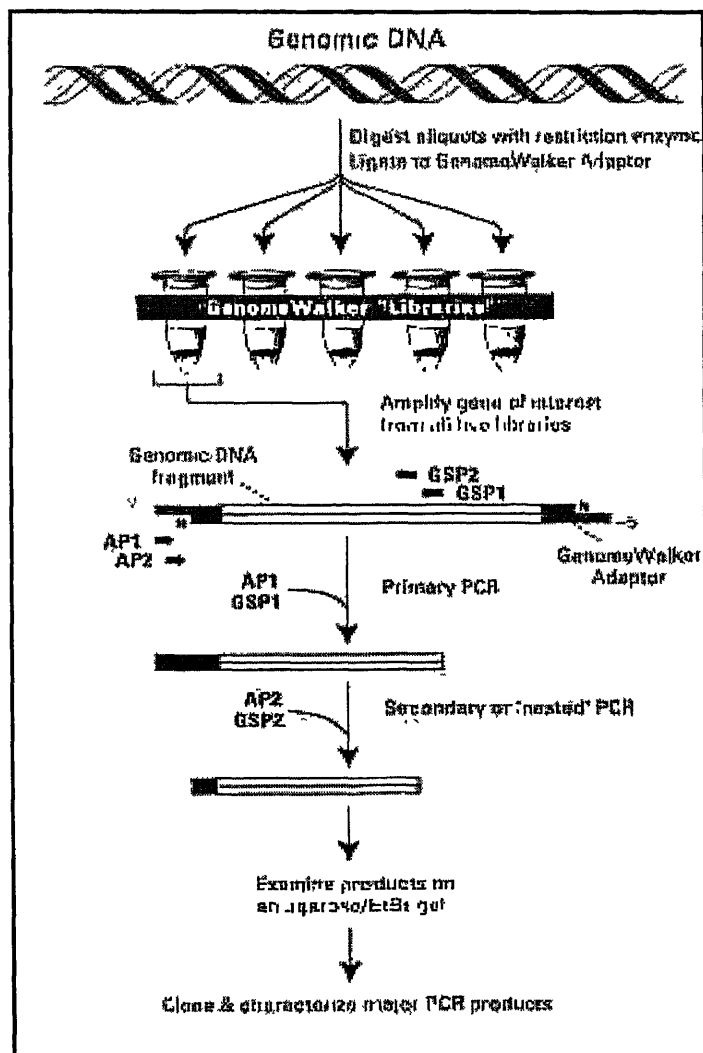


Fig. 5: Schematic Diagram of PCR Walking technique

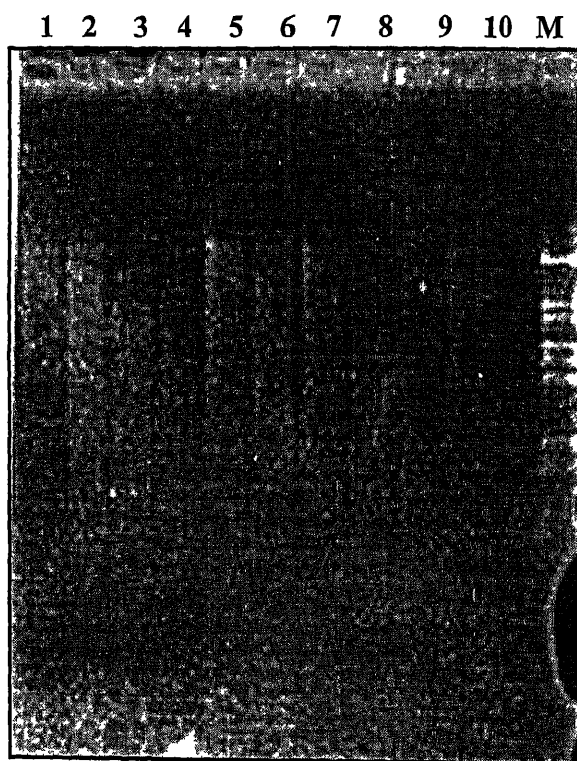


Fig. 6: Restriction digestion of *Arabidopsis* and *Brassica juncea* with different restriction enzymes. Lanes 1 to 5 show the *Arabidopsis* and lanes 6 to 10 show *B.juncea* DNA digested with *Eco* RV, *Dra* I, *Pvu* II, *Sca* I and *Ssp* I respectively. M represents marker lane.

Check 5 µl of digested sample on a 1% agarose gel to ensure complete digestion(Fig 6).

Extract the remaining sample once with phenol. chloroform: IAA (25:24:1) and once with chloroform:IAA (24:1).

Precipitate the DNA by adding 1/10 volume of 3M sodium acetate (pH-4.8) and two volumes of ethanol (chilled) or incubate at -20°C for 10-15 min

Centrifuge in a microfuge for 10 min. Wash the pellet with 70% ethanol. Dry and dissolve in 10 µl sterile water.

i **Adaptor ligation**

Each batch of digested genomic DNA is ligated separately to the adaptor. However, before that you need to make the adaptor duplex by annealing together single-stranded oligo I and oligo II. This produces an adaptor duplex, which has one blunt end for ligation and one amino end to prevent extension.

Adaptor Oligonucleotide 1:

5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCCGGGGAGGT-3' and

adaptor oligonucleotide 2: 5'-P-ACCTCCCC-NH₂-3' were mixed to a final equimolar concentration of 50µM and annealed by incubation in a boiling water-bath for 5 min followed by gradual cooling to allow formation of the adaptor duplex.

iii. **Making of adaptor duplex**

- ☐ Mix two adaptors so that the final concentration is 50 µM.

Oligo I (100 pm/µl) = 12.5 µl

Oligo II (100 pm/µl) = 12.5 µl

= 25.0 µl (final conc. is 50 µM)

Put the tube containing the mix in boiling water for 10 min. and let it cool to room temperature.

iv. **Ligation**

To each of the four tubes of digested DNA add the adaptor duplex and keep for ligation.

Add the following (5 sets)

Digested DNA	10.0 µl
10 X ligase buffer	2.0 µl (Boehringer)
T4 DNA ligase	1.0 µl
Adaptor duplex	2.0 µl (Final conc. will be 5 µM)
Water	5.0 µl

	20.0 µl

- Incubate overnight at 16°C.
- Heat inactivates the T4 DNA ligase by heating at 70°C for 10 min.
- Add 180 µl of water to make the final volume to 200 µl and store at -20°C. (These 5 sets of reactions constitute the genomic library).

v. PCR amplification of genomic library for a target region

The adaptor ligated DNA is used as a template for two nested PCR amplification reactions using adaptor primers and gene-specific primers. The adaptor primers are shorter in length than the adaptor and are capable of hybridizing to the outer primer-binding site.

Two sets of amplifications are done one after another.

vi. Primers used

AP1	5'-GGATCCTAATACGACTCACTATAGGGC-3' (27 mer)
SS1:	5'-GTTGGCTGTGGCCGCGGTTATCA-3' (24 mer)
AP2 :	5'-CTATAGGGCTCGAGCGGC-3' (18 mer)
SS2 .	5'-GCCATTGATCCAAAAGAGACGCAG-3' (24 mer)

vii. Ist PCR amplification

The Ist amplification makes use of primer API (adaptor primer) and primer SS1 (gene-specific primer).

For each set, add the following.

DNA-Adaptor complex	1.0 µl
10 X Taq Buffer	0.4 µl
(Boehringer) High Fidelity - Taq Polymerase (3U/µl)	0.4 µl
10 mM dNTPs	0.4 µl
Primer 1 (API)	0.4 µl
Primer 2 (SS1)	0.4 µl
Water	15.4 µl

	20.0 µl



Fig. 7: PCR amplification products obtained after PCR I and PCR II and separated on 1% agarose gel. Panel A, lanes 1 to 5 shows the PCR I products of *B. juncea* from PCR Walking libraries obtained after *Eco* RV, *Dra* I, *Pvu* II, *Sca* I and *Ssp* I digestion. Panel B, lanes 1 to 5 shows the PCR products obtained after PCR II from different PCR walking libraries. M is marker lane. The specific product obtained marked by an arrow is taken for cloning.

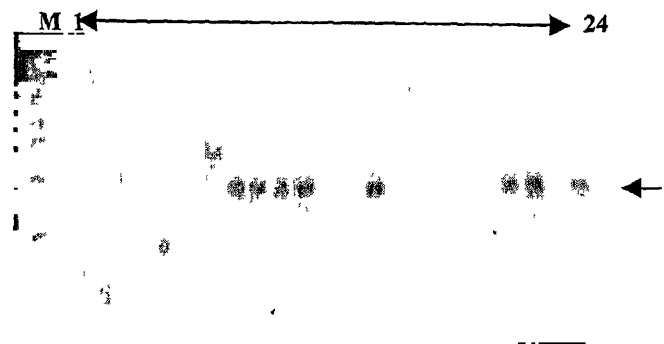
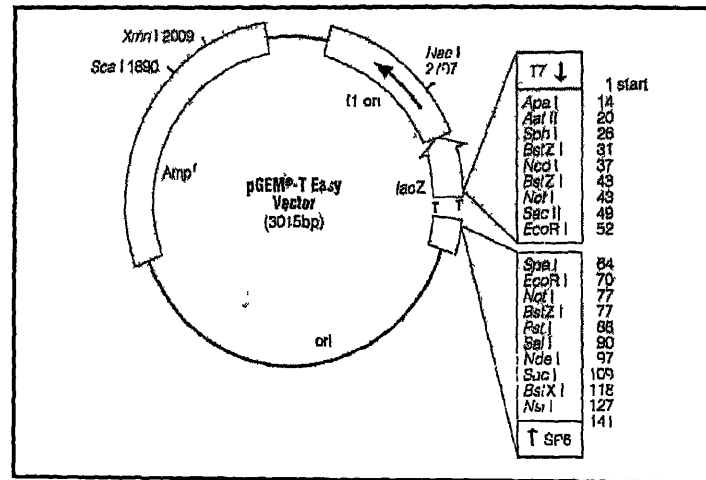


Fig. 8: Panel A, shows the plasmid vector map of pGemTeasy vector. Panel B, 1 to 24 shows the colony PCR of the recombinant clones obtained after cloning of sucrose synthase promoter in pGemTeasy vector. M is marker lane (1 kb ladder). Arrow shows the right size of the insert.

Add one drop of mineral oil and PCR amplify using the two step PCR with the following parameters:

Denaturation	94°C - 1 min	} 7 cycles
Annealing/Extension	72°C - 4 min	

Denaturation	94°C - 1 min	} 32 cycles
Annealing/Extension	65°C - 4 min	

10 µl of the first PCR amplification products were loaded onto 1% agarose gel and electrophoresed for one hour and viewed after ethidium bromide staining. It is clear from the **figure 7A** that we obtained only smear after first PCR.

Dilute the remaining 10 µl of the first PCR reaction product to a final volume of 100 µl.

viii. 2nd PCR amplification

The 2nd PCR amplification makes use of the nested primer AP2 (adaptor primer) and the SS2 primer (gene-specific primer).

For each set, add:

DNA (Diluted from 1st PCR)	1 0 µl
10 X PCR buffer	2.0 µl
(Boehringer) Expand long - Taq Polymerase	0.4 µl
10 mM dNTPs	0.4 µl
Primer 3 (AP2)	0.4 µl
Primer 4 (SS2)	0.4 µl
Water	15.4 µl

	20.0 µl

Add one drop of mineral oil and PCR amplify using the cycling parameters of the 1st PCR. Load 5 µl on a 1.2% agarose gel. The results of the 2nd. PCR are shown in (**Figure 7B**). In the second PCR we have obtained specific fragments which differ for the various restriction enzymes as seen in **Figure 7B**. It is clear that for *Pvu* II and *Ssp* I we obtained the larger size fragments approximately 1.0kb in length and hence these were taken for cloning and characterization.

The specific fragments obtained after second PCR were cloned in pGemT-easy vector (Promega, USA) and transformed in E.coli JM109 strain.

The *E.Coli* colonies were screened for insert by colony PCR amplification method using vector specific primers namely SP6 and T7 primers(**Figure 8A**). It is clear from colony

PCR (**Figure 8B**) that the recombinant clones contained mostly the inserts of right size depending on the enzymes used for PCR walking.

The Plasmid DNA was isolated from recombinant clones and sequenced by automated sequencer using fluorescently labeled ddNTPs (**Fig. 9**). The sequence homology was carried out using the EMBL database (fig10). It was confirmed that the sequence resembles with the reported sucrose synthase like sequences in the databank.

Direct amplification of sucrose synthase promoter using PCR primers derived based on the sequence of *Arabidopsis thaliana*:

The oligonucleotide sequences were designed from the promoter region of the reported sequence of sucrose synthase of *Arabidopsis thaliana* (**Fig.10**). The primers named as SSF1 and SSF2 were designed upstream of the promoter sequence while SSR3 downstream of the promoter region. Amplified fragment from genomic DNA of *Arabidopsis thaliana* (columbia), were cloned in pGem T-easy vector and transformed in *E. coli* strain JM-109. The recombinant plasmids were screened for the desired insert by colony PCR method using vector specific primers. The orientation of the cloned fragments were determined by using one vector specific primer and on common primers SSR3 which is designed just upstream of ATG site of the promoter. The following clones were identified in right orientation from *B.juncea* and *Arabidopsis thaliana*.

1. Sucrose synthase from *B.juncea*. SS1, SS2, SS4, SS6 and SS8 (0.9 Kb)
2. Sucrose synthase from *A.thaliana* SSF2-1, SSF2-2, SSF2-3, SSF2-4 and SSF2-7 (0.9 Kb).
3. Sucrose synthase from *A.thaliana* SSF1-33.

Since the size of the sucrose synthase SSF1-33 is large it was not used as a promoter sequence.

The promoter sequence was analysed for the different motifs. The various conserved motifs are shown in the table below.

>EM_PL:ATSUS1 X70990 A.*THALIANA* GENE FOR SUCROSE SYNTHASE

Length = 5007

Score = 1054 (164.2 bits), Expect = 1.8e-40, P = 1.8e-40

Identities = 254/284 (89%), Positives = 254/284 (89%), Strand = Minus / Plus

```
Query      303 AACCTGGAAG--TGAA-GC-TAAAAGAAAAGAAAAGCATGCCAATAAAATT-CTTG-CTTT 250
          || || |||| | || || ||||| |||| | | ||||| |||| ||||
Sbjct.    1269 AAGCTTGAAGGCTAAAAGCGTAAAAGAAAAGTCATGTTTTC-AATAAATTCCTTGACTTT 1327

Query:     249 TAGTTATAATA-CTTGACCTGTTGTTATAATATCTAACCTATAGTTT-CTTTATATATA 192
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct.    1328 TAGTTATAATATCTTGACCT-TTGTATAATATCTAACCTATAGTTTTCTTTATATATA 1386

Query:     191 ATGTGAATATGGAAAACACAAGACTTCATATCTTATTTTCATCAATCTTATTCTTATTTT 132
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct.    1387 ATGTGAATATGGAAAACACAAGACTTCAT--CTTATATTCATCAATCTTATTCTTATTTT 1444

Query.     131 CTCTCTGCAGTTTTCAAAAACCTTCTCACTTTTTTCTGCGTCTCTTTTTTTGAATCAATG 72
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct.    1445 CTCTCTGCAGTTTTCAAAAACCTTCTCACTGTTTTCTGCGTCTCTTTTG--GA-TCAATG 1501

Query.     71  GCAAACGCTGAACGTATGATAACGCGCGTCCACAGCCAAA-TGA 29
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct.    1502 GCAAACGCTGAACGTATGATAACGCGCGTCCACAGCCAACGTGA 1545
```

Fig.9: Homology search of the cloned sucrose synthase promoter with the sequences in EMBL database. Query represents the SS cloned and Sbjct represents the sequence in the database.

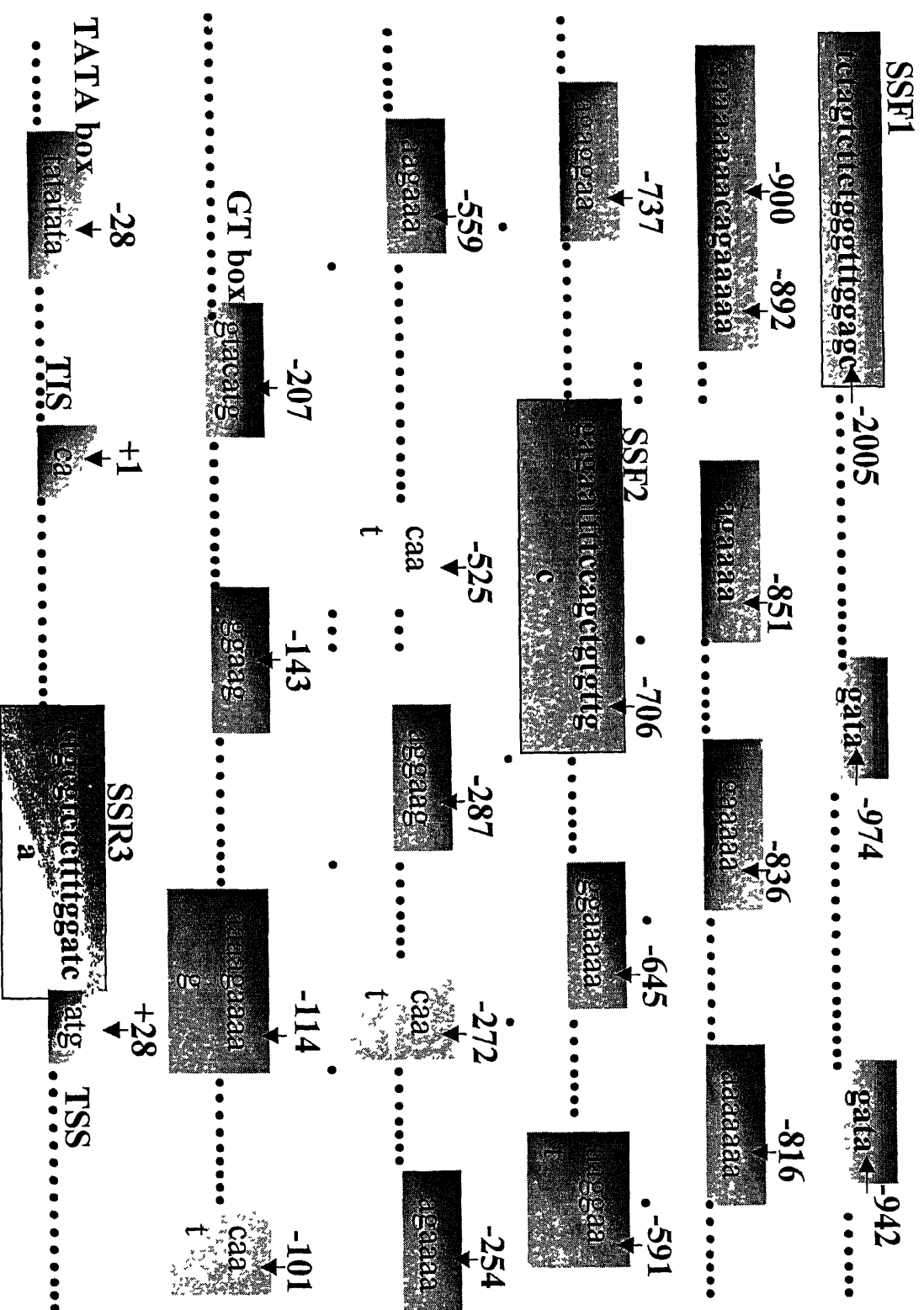


Fig. 10 The sucrose synthase promoter was analysed for its motifs. The TIS, TATA box, CAA box are shown. Purine rich elements are also interpreted. The different primers SSF1, SSF2 and SSR3 are shown in the boxes.

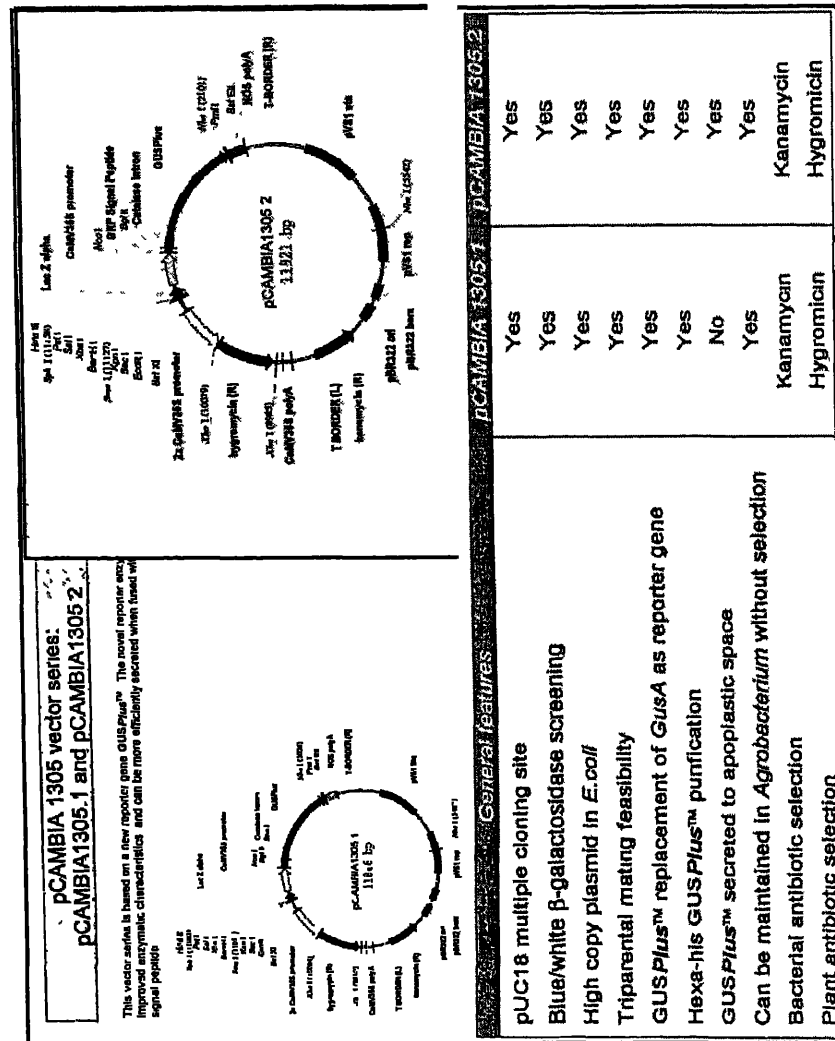


Fig. 11A Vector maps of *Agrobacterium* binary plasmid pCambia1305.1 and pCambia1305.2 and their important features.

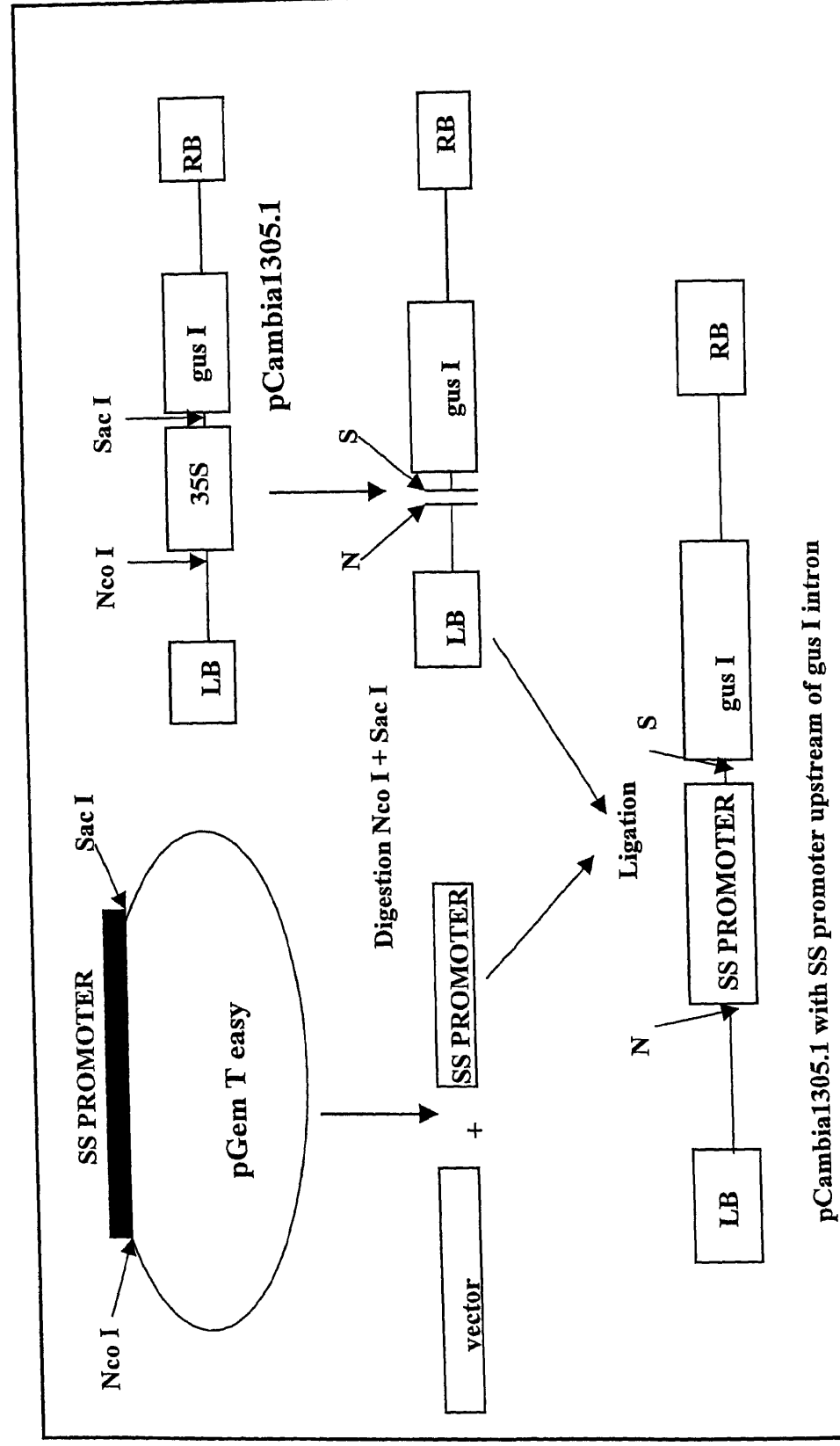


Fig.11B : Strategy for cloning of sucrose synthase promoter in pCambia1305.1 upstream of gus intron gene

Conserved motif	Position with respect to	Position in the
	+85	1499 – 1501
TIS	-1 to +1	1414 – 1415
TATA-Box	-28 to -35	1379 – 1387
TSS	-101 to -104	309 – 312
	-268 to -272	1142 – 1145
	-287 to -290	1125 – 1128
	-525 to -528	885 – 889
Purine rich	-114 to -123	1268 – 1273
	-143 to -147	1289 – 1298
	-249 to -254	1161 – 1166
	-353 to -359	1064-1070
	-559 to -564	850 – 855
	-591 to -597	817 – 823
	-645 to -551	763 – 769
	-737 to -743	670 – 676
	-812 to -816	591 – 597
	-836 to -841	573 – 578
	-851 to -856	558 – 563
	-892 to -898	516 – 522
	-900 to -908	506 - 514
GT like elements	-207 to -213	1201 – 1208
GATA box	-942 to -945	469 – 472
	-974 to -977	437 - 440

Subcloning of promotor in *Agrobacterium* plasmids:

The clones from sucrose synthase were further subcloned in pCambia –1305 plasmid (Fig.11A) vector for expression of *gus* intron gene for use as a phloem specific promoter. The strategy for cloning is given in figure 11B.

Steps:

- The plasmid DNA from pCambia-1305 was digested with *Nco* I + *Sac* I to delete the 35 S CaMV promoter.
- The plasmid DNA from SS and SSF2 clones were also digested with same restriction enzymes.
- The sucrose synthase fragments and the plasmid fragments minus the 35s promoter were ligated and transformed in *E.coli* JM109 cells.
- The transformed *E. coli* colonies were screened for the insert of right size by colony PCR method.
- Plasmid DNA was isolated from the recombinant colonies and these are further mobilized into *Agrobacterium* strains A4404 and EHA-105.

Cloning and characterization of lectin gene in plasmid and *Agrobacterium* binary vectors

Steps:

- a. The cDNA clone named LecGNA was first digested with *Hind* III and partially digested with *Eco* RI to obtain the right size fragment. (**Figure 12**, lane 3)
- b. The plasmid vector pGem7Zf was digested completely with *Hind* III and *Eco* RI enzymes. (Figure 12A, lane 1 & 2)
- c. The Lec GNA fragment was partially digested with *Eco* RI, the fragment of size 470bp and the fragment with *Hind* III digest of size 570 bp were eluted from the agarose gel. (See figure 12A, lane 4 to 6)
- d. The vector was digested with *Hind* III and *Eco* RI was separately ligated with the Lec GNA fragments obtained after *Hind* III and *Eco* RI respectively.
- e. The plasmid containing the insert was transformed in E coli DH-5 α strain.
- f. The recombinant plasmids were screened for the presence of insert by PCR amplification and restriction digestion of the recombinant plasmids with *Eco* RI (Figure 12B). It is clear from the figure that the lectin gene has been cloned into pGem7Zf vector as it shows the presence of the both vector fragment (approx 3 kb) and lectin gene fragment ($\cong$ 500bp).
- g. Restriction mapping of recombinant plasmids for orientation of the insert in plasmid vector was undertaken. For orientation experiments, 4 recombinant (namely 1, 2, 24 & 25) clones were mapped using differentiation enzymes such as *Xba* I, *Xho* I, *Eco* RI, *Cla* I, *Hind* III and *Bam* HI (**Figure 13**). It is clear from *Hind* III restriction that clones 24 and 25 are in the reverse orientation. Thus these two clones were taken up for detailed characterization and analysis.
- h. The two clones # 24 and #25 were sequenced using the automated ABI prism using fluorescently labeled ddNTPS. The partial sequence of the recombinant lectin clone in pGem72F is shown in **Figure 14**. Similarly, the sequence of recombinant clone # 25 is shown in **Figure 15**. The sequences were undertaken for analysis using the different software available in public domain. The completed sequences were compared with the database to confirm whether the sequences were correct or not. Figure 16 shows the homology for sequence shown in query and compared with lectin gene in the database shown as subject. It is clear from **Fig. 16** that we have the sequences in the right orientation in the plasmid vector(Clone#25) which is necessary for expression of gene in the phloem.

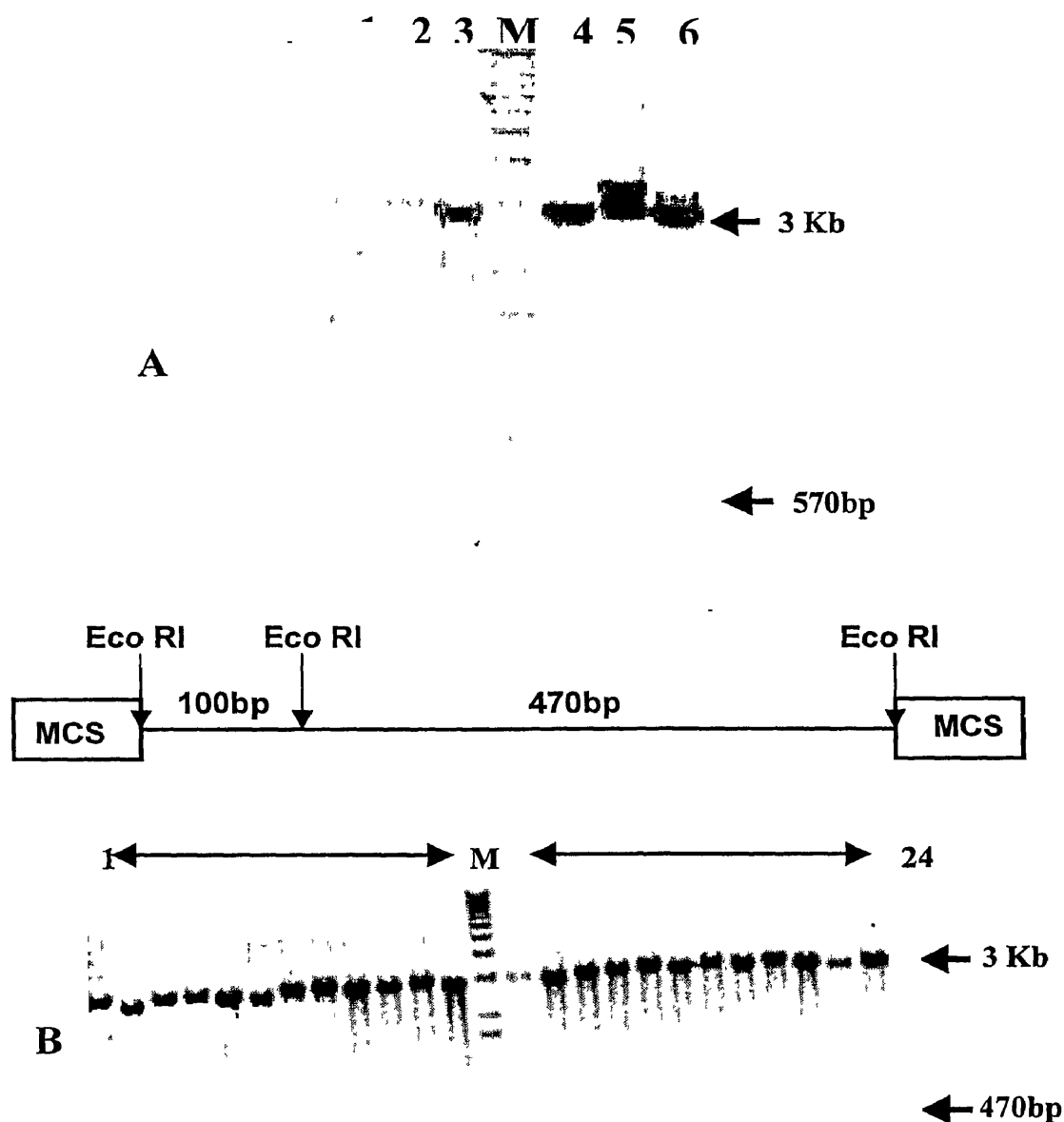
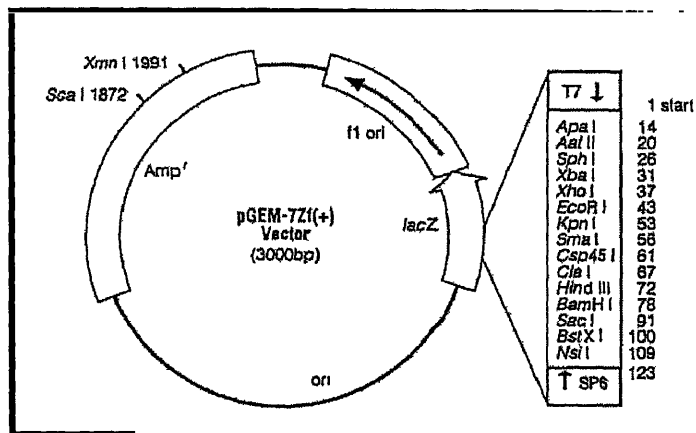


Fig.12: A , Lane 1&2 represent plasmid vector pGem7zf digested with *Eco* RI and *Hind* III, lane 3 LecGNA digested with *Hind* III. M is marker and lanes 4,5 and 6 are clone LecGNA digested with *Eco* RI. B, 1 to 24 represents the digestion of recombinant plasmids with *Eco* RI. Arrows show the size of the plasmid and the insert.



Orientation analysis

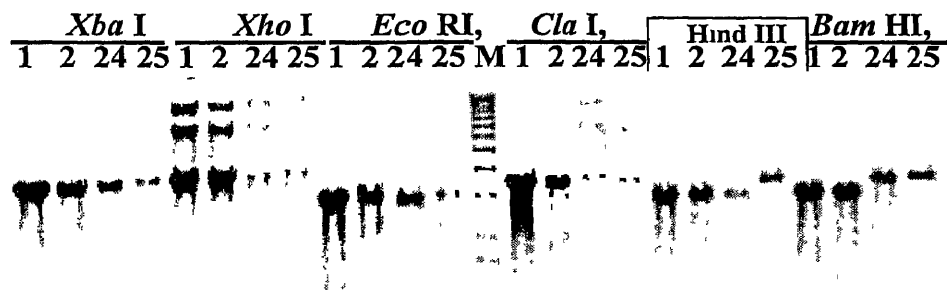


Fig.13

A , represents the map of plasmid vector pGem7zf. B, 1,2, 24 and 25 represent the four recombinant clones digested with *Xba* I, *Xho* I, *Eco* RI, *Cla* I, *Hind* III and *Bam* HI. *Hind* III digest clearly shows that clones 24 and 25 are in the reverse orientation. M is marker(1kb ladder).

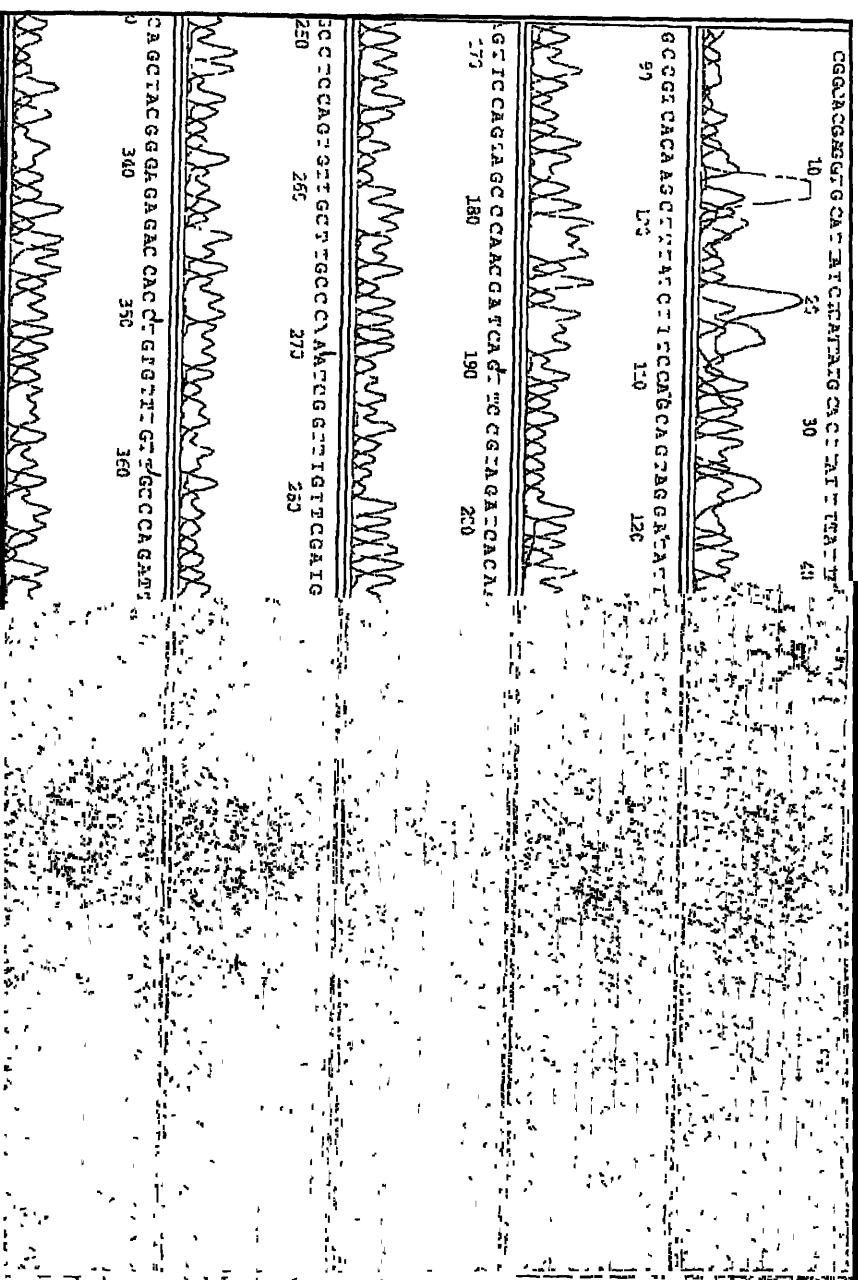


Fig. 14: Electrophorogramme of the c1one pLec 24 obtained after automated sequencing

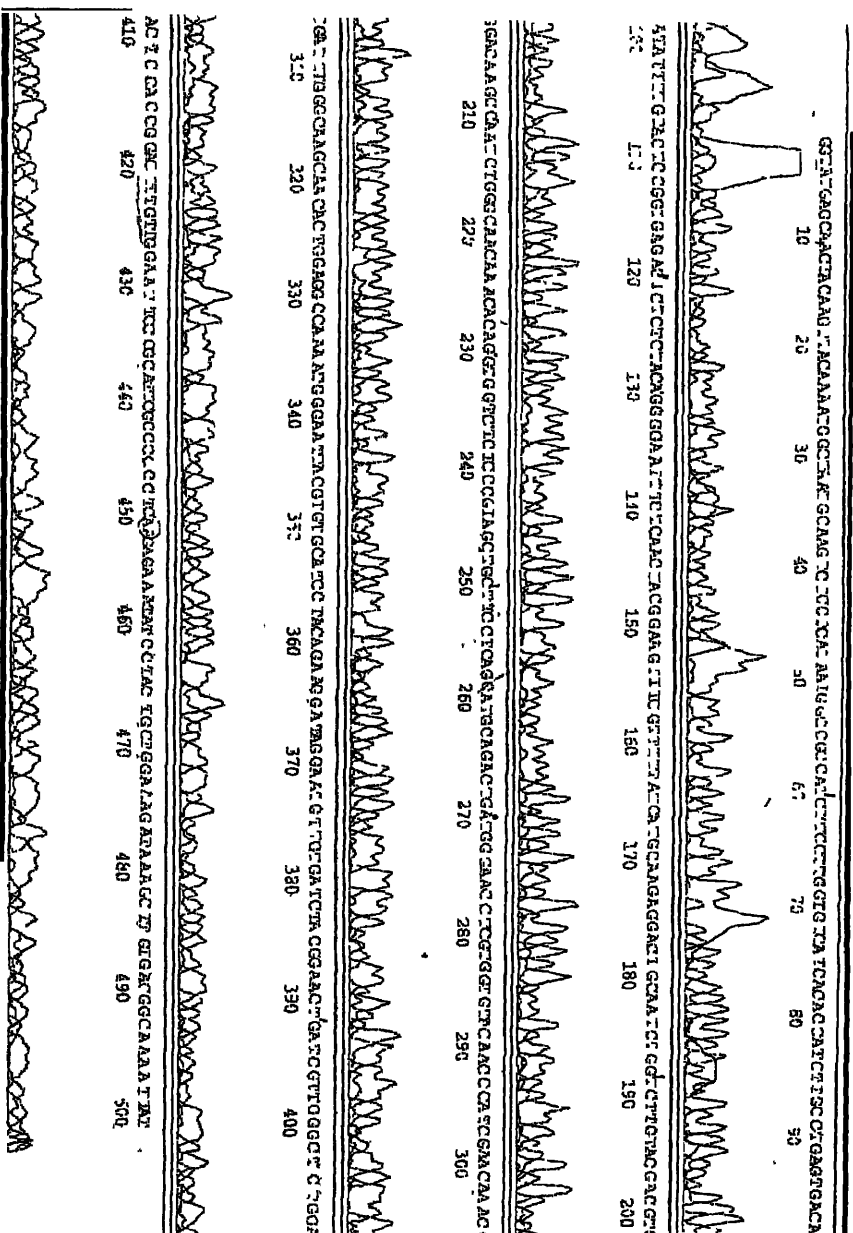


Fig. 15: Electropherogramme of the clone pJec25 obtained after automated sequencing

GenBank">gb|M55556.1|GAAL2A *Galanthus nivalis* lectin
 (LECGNA 2) mRNA, complete cds
 Length = 570
 Identities = 480/490 (97%), Gaps = 6/490 (1%)
 Strand = Plus / Plus

```

Query. 9   caactacaagttacaaaatggctaagtgaagttctctcataatggccgcatcttccttg 68
          |||
Sbjct. 1   caactacaagttacaaaatggctaaggcaagttctctcattttggccgcatcttccttg 60

Query. 69   gtgtcatcacaccatcttgctgagtgacaatatatttgactccggtgagactctctcta 128
          |||
Sbjct. 61   gtgtcatcacaccatcttgctgagtgacaatatatttgactccggtgagactctctcta 120

Query. 129   caggggaatttctcaactacggaagtttcggtttttatcatgcaagaggactgcaatctgg 188
          |||
Sbjct. 121   caggggaatttctcaactacggaagtttcggtttttatcatgcaagaggactgcaatctgg 180

Query. 189   tcttgtagcagctggacaagccaatctgggcaacaaacacaggtggtctctcccgtagct 248
          |||
Sbjct. 181   tcttgtagcagctggacaagccaatctgggcaacaaacacaggtggtctctcccgtagct 240

Query. 249   gcttcctcagcatgcagactgatgggaacctcggtggtgtacaacccatcgaacaaaccg- 307
          |||
Sbjct. 241   gcttcctcagcatgcagactgatgggaacctcggtggtgtacaacccatcgaacaaaccga 300

Query. 308   tttgggcaagcaacactggaggccaaaatgggaattacgtgtgcatcctacagaaggata 367
          |||
Sbjct. 301   tttgggcaagcaacactggaggccaaaatgggaattacgtgtgcatcctacagaaggata 360

Query. 368   ggaatgttgtgatctacggaactgatcggtgggct-ctggaactc-caccggactttgtt 425
          |||
Sbjct. 361   ggaatgttgtgatctacggaactgatcggtgggctactggaactcacaccggac-ttgtt 419

Query. 426   ggaattcccgcatctcgccccctcaagagaaatatcctactgctggaaagataaagcttg 485
          |||
Sbjct. 420   ggaattcccgca-tcgccaccctc-agagaaatatcctactgctggaaagataaagcttg 477

Query. 486   tgacggcaaa 495
          |||
Sbjct. 478   tgacggcaaa 487

```

Fig.16: Homology search of lectin sequence with EMBL database. Query is the sequence of clone pLec25 and sbjct being the sequence of *Galanthus nivalis* lectin (LECGNA 2) mRNA.

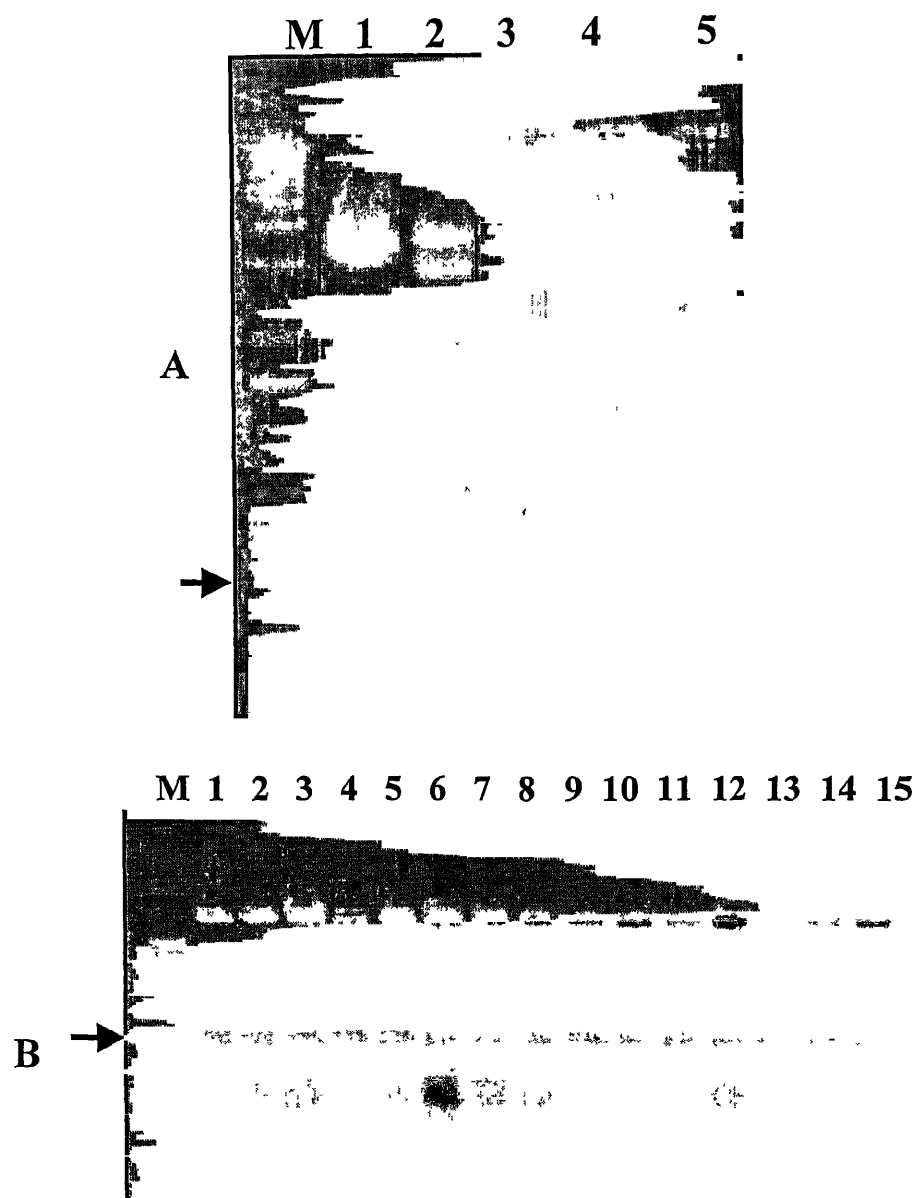


Fig. 18:

Panel A , Lane 1 and 2 show plasmid pLec 25 digested with *Hind* III and (*Xba* I + *Sac* I). Lane 3, 4 and 5 are the Agrobacterium plasmids pE1226, pE1774 and pE1780 digested with (*Xba* I + *Sac* I.) respectively. Panel B, lanes 1 to 15 show the recombinant plasmids amplified with Lectin primers (LecF and LecR) by colony PCR method. M is marker (1kb ladder). Arrows show the size of the insert cloned and PCR amplified.

2 Cloning of the Lectin gene fragment in Agrobacterium binary vector.

The plasmid Lec-25 was further sub-cloned in following Agrobacterium plasmids namely :

- i) pBI 121
- ii) pBISNI(E-1226)
- iii) pBISNI(E-1774)
- iv) pBISNI(E-1780)

The vectors containing super-promoters namely, pE1226, pE1774 and pE1780 are shown schematically in **Figure 17**. All the three vectors have super promoter (AoCS)₃ but have different selection markers. In pE1226 (**Figure 17A**), the vector contains kanamycin gene driven by nos promoter. It also contains the gus intron gene driven by super promoter. Similarly the vector pE1774 (**Figure 17B**) has hygromycin gene and pE1780 (**Figure 17C**) has bar gene for plant selection.

We decided to use these super promoters for plant transformation as one can expect higher levels of gene expression as compared to 35S constitutive promoter. The following steps were taken to sub-clone the lectin gene fragment from pGem7Zf vector to Agrobacterium binary vector:

- a. Plasmid pGLec25 was digested with restriction enzymes *Xba* I + *Sac* I to take out the insert from the plasmid. We could not use *Eco* RI as it has a site in the gene fragment.
- b. The Agrobacterium plasmids pBI121, pE1226, pE1774 and pE1780 were also digested with *Xba* I + *Sac* I to delete the gus intron gene from the plasmid(**Fig.18A**) .
- c. The fragments Lec-25 and agrobacterium plasmid fragment (minus gus) were eluted from the gel and ligated using T4 DNA ligase.
- d. The ligated DNA was transformed in *E. coli* strain DH-5 α .
- e. The recombinant plasmids were screened for insert by using Vector specific or lectin primers in colony PCR method (**Figure 18B**).

These plasmids are further mobilised into Agrobacterium strains A4404 and EHA105 for plant transformation.

II Genetic Transformation of *Brassica juncea*

For the successful transformation of *B. juncea*, we have to undertake regeneration studies using different explants. We were facing problems using hypocotyle explants for genetic transformation. So we wanted to look at other explants such as cotyledonary petiole, cotyledons etc which have been reported to be excellent explants for genetic transformation in *B. napus*. Thus studies on regeneration of *B. juncea* were undertaken using cotyledonary petiole and cotyledon explants.

In vitro regeneration

Plant Material

Brassica juncea var. Pusa Jaikisan were selected for present study because of its commercial importance. Seeds from the variety were used for initiation of cultures

Surface disinfection of seeds and seed germination

Seeds were treated with bavistin solution (2-5%) and 2-3 drops of Tween 20 for 20 min, followed by repeated rinses in tap water for 30 min. Surface disinfection was carried out with 0.1% mercuric chloride for 8 min, followed by 3-5 rinses in sterile distilled water. Seeds were germinated on half strength MS medium (Murashige and Skoog, 1962) solidified with 0.8% agar (Hi Media) and pH adjusted to 5.8 prior to autoclaving, in 500 ml cultures jars (Table 4). They were implanted aseptically at a density of 20-30 seeds per culture jar, to allow for uniform growth of seedlings. Germination was carried out at 23 °C in a 16h light and 8 h dark photoperiod, at a light intensity of 1500 lux.

Preparation of explants

Cotyledons with 1-2 mm petiole were excised from 5-day-old seedlings (unless otherwise stated). Care was taken to remove apical buds present at cotyledonary axis. Cotyledons were planted on the regeneration medium with their abaxial surface in contact with medium and the cut petiole embedded in the medium. Ten cotyledons were cultured per 15 x 90-mm petridish, containing 20-25 ml of the shoot regeneration medium.

Table 4. Composition of Murashige and Skoog medium

Constituents	Concentration in culture medium (mg l ⁻¹)
Macronutrients	
KNO ₃	1900
NH ₄ NO ₃	1650
CaCl ₂ 2H ₂ O	440
MgSO ₄ .7H ₂ O	370
KH ₂ PO ₄	170
Micronutrients	
MnSO ₄ .H ₂ O	16.9
ZnSO ₄ 4H ₂ O	8.6
KI	0.83
Na ₂ MoO ₄ .2H ₂ O	0.25
CoCl ₂ 6H ₂ O	0.025
CuSO ₄ 5H ₂ O	0.025
Iron source	
Na ₂ EDTA	37.3
FeSO ₄ .7H ₂ O	27.8
Organic supplements	
Amino acids	
Meso-Inositol	100
Glycine	2.0
Vitamins	
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Thiamine-HCl	0.5
Carbon source	
Sucrose (%)	0.3
Complex addenda	
Agar (%)	0.8

Regeneration

Conditions for *in vitro* shoot regeneration from the cotyledonary petiole of *B. juncea* var. PJK were optimized.

Optimization of medium for shoot regeneration

To investigate the capacity of shoot regeneration of cotyledonary petiole explant of *B. juncea* var. PJK, 5-day-old aseptically germinated seedlings were used. Explants were

cultured on MS medium supplemented with (0.5 to 6.0 mg/l) BAP in petri-plates. Inclusion of growth hormones e.g. cytokinins and auxins is necessary to support regeneration of shoots from the explants. However, requirement of substances varies considerably with plant species and explant type. In the present study, explants were cultured on MS medium supplemented with various concentrations (0.0, 0.5, 1.0, 2.0, 2.5, 3.0, 4 and 6.0 mg/l) of BAP (**Fig. 19**). A total of 40 explants were taken for each experiment and the data was recorded after 4 weeks of culture (**Table 2**). Data was recorded in terms of the percentage of explants regenerating shoots and the number of shoots formed per explant.

Presence of BAP in the medium was found to be a critical factor for shoot regeneration. In absence of BAP there was no induction of shoot regeneration. At BAP concentrations of 0.5 and 1.0 mg/l regeneration frequency of 22.14% and 28.57% was obtained. Maximum shoot regeneration (53.57%) was observed at a BAP concentration of 2.5 mg/l. Only 30.25% of the plants regenerated on 3.0 mg/l of BAP. At higher concentrations of BAP (more than 3.0 mg/l), there was a sharp decline in shoot morphogenesis (data not presented). BAP at the concentration of 2.5 mg/l also supported formation of maximum number of shoots (5.0) per explant. Only 1 or 2 shoots were formed per explant at other BAP concentrations.

Effect of different concentrations of AgNO_3 (0 to 50 μM) was also studied. Inclusion of ethylene inhibitor such as AgNO_3 in the shoot regeneration medium has been shown to strongly influence the shoot regeneration frequency. Explants were therefore cultured on MS

Table 2. Effect of BAP and AgNO₃ on *in vitro* shoot regeneration from cotyledonary petiole of *B. juncea* var PJK, after 4 weeks of culture

BAP (mg/l)	AgNO ₃ (μM)	No. of explants	Regeneration (%)	No. of shoots formed per explant
0.0	0	40	0.0	0.0
0.5	0	40	22.14	1.09
1.0	0	40	28.57	1.98
2.5	0	40	53.57	5.54
3.0	0	40	30.28	0.5
0.0	10	40	0.0	0.0
0.5	10	40	35.70	2.57
1.0	10	40	51.42	3.4
2.5	10	40	67.85	7.12
3.0	10	40	21.2	2.25
0.0	20	40	0.0	0.0
2.5	20	40	92.57	14.3
3.0	20	40	62.1	2.7
0.0	30	40	0.0	0.0
0.5	30	40	27.14	1.19
1.0	30	40	52.85	3.0
2.5	30	40	71.42	6.1
3.0	30	40	32.11	3.2
0.0	50	40	0.0	0.0
0.5	50	40	14.28	1.57
1.0	50	40	39.85	2.46
2.5	50	40	60.42	4.52
3.0	50	40	22.9	2.1

\medium containing different concentrations of BAP (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 6.0 mg/l) and supplemented various concentrations (0, 10, 20, 30, 50 μM) of AgNO₃. More than 40 explants were taken for each experiment and the data was recorded after 4-weeks of

culture (**Table 2**). In the absence of BAP, AgNO₃ could not induce shoot regeneration. At a BAP concentration of 2.5 mg/l shoot regeneration frequency was observed to increase from 53.57% to 92.57% on inclusion of AgNO₃ (20 µM). Also at this concentration of AgNO₃, number of shoots formed per explant increased from 5.0 to 14.0 (**Fig.19**). Silver nitrate at a concentration higher 20 µM proved to be inhibitory to shoot morphogenesis.

One cm long shoots were obtained after 4 weeks of culture on MS medium containing optimum level of BAP (2.5 mg/l) and AgNO₃ (20 µM). The shoots were green and robust. These concentrations of BAP and AgNO₃ were therefore used in all our experiments.

Different gelling agents (Difco agar; Hi media Agar and Agar gel, Sigma) were tested for their effect on shoot regeneration. Different gelling agents were therefore included in the culture medium at various concentrations (0.2%, 0.4%, 0.8%, 1.0%) in order to determine their effect on regeneration (**Fig 20**). After 4 weeks of culture, data was recorded in terms of the presence or the absence of shoot regeneration and the type of shoots formed (**Table 3**).

Table 3. Effect of various gelling agents on *in vitro* shoot regeneration from cotyledonary petiole of *B. juncea* var. PJK, after 4 weeks of culture.

Concentration of the gelling agent (%)	Agar gel (Sigma)	Hi Media agar	Difco agar
0.2	-	+	+
0.4	+	++	++
0.8	-	++++	++
1.0	-	+++	++

(-) No regeneration; (+) Vitrified, stunted shoots; (++) Vitrified, 1cm long shoots;

(+++ Non vitrified, stunted shoots; (++++ Green, robust shoots

Inclusion of Agar gel and Difco agar in the medium resulted in hyperhydration of the explant. No such effect was observed with Hi Media agar (0.8%). In presence of Agar gel (0.4%) shoots obtained were vitrified and stunted (**Fig 20**). Difco agar (0.8%) supported formation of green non-vitrified shoots, but these shoots were also stunted. Shoots obtained on Hi Media (0.8%) agar were green and robust (**Fig 20, Table 3**). At Hi Media agar concentrations other than 0.8%, shoot number declined. In all the experiments performed



Fig. 19. Effect of different concentrations of BAP on regeneration

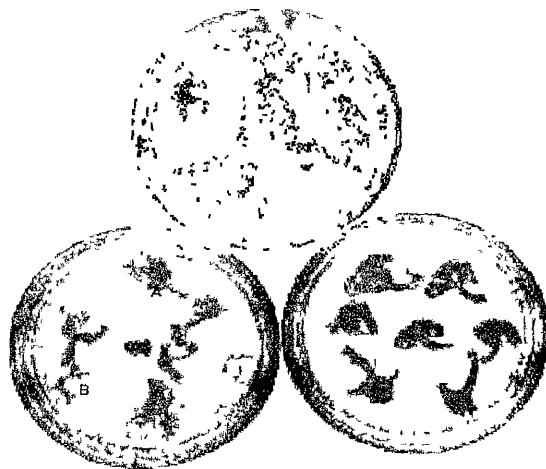


Fig. 20. Effect of different gelling agents on in vitro regeneration of *B. juncea* (A)-agar gel (Sigma) (B)-Hi media agar, and (C)- Difco agar

thereafter, H₁ Media agar (0.8%) was used as the solidifying agent (**Table 3**) as it gave the best results.

To improve the efficiency of *in vitro* regeneration, effect of explant age was evaluated. In all the above experiments, data was recorded after 4 weeks of culture. Age of the seedling from which the explant is derived has been shown by many workers to greatly influence the shoot regeneration potential. Therefore, in order to determine the effect of the explant age on *in vitro* shoot regeneration potential, explants taken from 1 to 9-day-old seedlings were cultured on MS medium supplemented with 2.5 mg/l of BAP and 20 µM of AgNO₃. Data recorded after 4 weeks of culture is tabulated in **Table 4**.

An increase in regeneration frequency was observed with increase in the seedling age, (till 5-days) when explants were cultured on MS medium supplemented with BAP (2.5 mg/l) and AgNO₃ (20 µM). Cotyledonary petioles derived from 1, 2, 3, 4 and 5-day-old seedlings (**Fig. 21**) supported a regeneration frequency of 3.5%, 20.5%, 35.47%, 58.38% and 92.8%, respectively (**Table 4**). Also the number of shoots per explant increased from 1.0 shoot obtained in 2-day-old explants to 5.0 shoots from 5-day-old explants. Thereafter (after 5 days) a sharp decline in shoot regeneration frequency as well as the number of shoots formed per explant was observed (**Table 4**). Explants derived from 6, 7 and 8-day-old seedlings regenerated with frequencies of 60.5%, 42.2% and 19.2%, respectively. Only 1 or 2 shoots were obtained per explant. Explants derived from 9-day-old seedlings did not regenerate. Thus, for all further experiments explants derived from 5-day-old seedlings were used as these gave the desired regeneration (**Table 4**).

Table 4. Effect of explant age on *in vitro* shoot regeneration from cotyledonary petiole explants of *B. juncea* var. PJK, after 4 weeks of culture

Seedling age (days)	No. of explants	BAP (mg/l)	Percentage of explants forming shoots		No. of shoots formed per explant	
			In absence of AgNO ₃	In presence of AgNO ₃	In absence of AgNO ₃	In presence of AgNO ₃
1	40	2.5	2.52	3.5	0	0
2	40	2.5	15.2	20.5	1	1
3	40	2.5	25.57	35.47	1	2
4	40	2.5	38.48	58.38	2	4
5	40	2.5	62.85	92.8	5	15
6	40	2.5	28.00	60.5	1	2
7	40	2.5	5.5	42.2	0	1
8	40	2.5	5.0	19.2	0	1
9	40	2.5	0.0	0.0	0	0

It was further observed that inclusion of AgNO₃ in the MS medium supplemented with BAP (2.5 mg/l) could induce shoot regeneration at higher frequency in the explants derived from seedlings more than 5-day-old (**Fig. 21, Table4**) Regeneration frequency from 7-day-old explants was observed to increase from 5% to 42% on inclusion of AgNO₃ in the regeneration medium, though only one shoot was formed per explant (**Table 4**).

Optimization of medium for shoot elongation

After 4 weeks, regenerated shoots from different explants (approximately 1 cm long) were excised and subcultured on fresh medium. MS medium was supplemented with

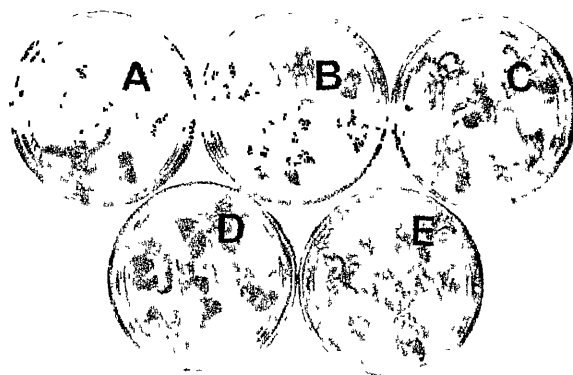


Fig. 21. Effect of different concentrations of AgNO_3 on in vitro regeneration (A)-0, (B) 10, (C)-20, (D)-30 and (E)-50 μM of AgNO_3

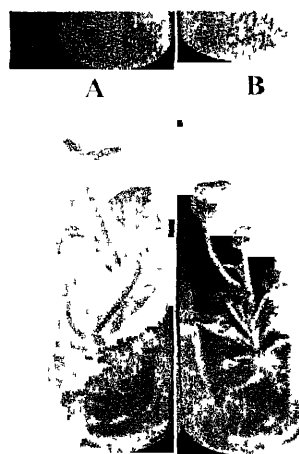


Fig. 22. In vitro rooting of *B. juncea* shoots on IBA supplemented medium (A) roots on MS supplemented with IBA (0.5 mg/l) (B) roots on MS basal medium

different concentrations of BAP (0, 0.1, 0.5, 1.0 and 2.5 mg/l) to see the effect on shoot elongation. Exposure of the regenerated shoots to high levels of cytokinins for prolonged periods has been shown to retard shoot elongation. Four week old regenerated shoots (1cm long) were therefore excised and subcultured on MS medium supplemented with lower BAP concentrations (0, 0.1, 0.5 and 1.0 mg/l). Data was recorded in terms of the length of shoot attained after 4 weeks of culture on this medium (**Table 5**). MS medium devoid of cytokinins (BAP) supported maximum shoot elongation. With increase in BAP concentration in the medium, length attained by the shoot declined from 4.5 cm long shoots obtained on 0.1mg/l of BAP to 2.0 cm long shoots obtained on 1.0 mg/l BAP. In presence of BAP (2.5 mg/l) shoot elongation was negligible even after 2 months of subculture.

Table 5. Effect of varying concentrations of BAP on elongation of shoots regenerated from cotyledonary petiole of *B. juncea* var. PJK.

BAP (mg/l)	Shoot length (cm)
0.0	6.0
0.1	4.5
0.5	2.5
1.0	2.0
2.5	1.0

Optimum medium for rooting

Presence of auxin in tissue culture medium is known to promote root induction. Elongated shoots (4.0-6.0 cm long) were therefore transferred to MS basal as well as MS medium supplemented with IBA (0.1, 0.5, and 1.0 mg/l;). After 3 weeks of culture, data was recorded in terms of the presence and the absence of intervening callus and the type of roots formed (**Table 6**). On MS supplemented with IBA, fibrous roots with intervening callus were formed. Size of the callus also increased with increase in IBA concentration. MS basal medium supported formation of non-fibrous roots (**Fig. 22**).

Table 6. Effect of various concentrations of IBA on formation of roots

IBA (mg/l)	Root type	Intervening callus
0	Non- fibrous	-
0.1	Fibrous	-
0.5	Fibrous	+
1.0	Fibrous	+

-Absent, + Present

Optimized conditions for regeneration

MS medium supplemented with BAP (2.5 mg/l), AgNO₃ (20 µM) and solidified with Hi Media agar (0.8%) was found to support a maximum frequency of *in vitro* shoot regeneration from the 5-day-old cotyledonary petiole of *B. juncea* var. PJK. After 4 weeks of culture on this medium, 1 cm long green and healthy shoots were obtained. These shoots elongated within 3 weeks of transfer to shoot elongation medium (MS basal). Elongated shoots formed healthy roots within three weeks of subculture on MS basal medium. After hardening on, plantlets were transferred to the fields. The protocol standardized for *in vitro* shoot regeneration from *B. juncea* var. PJK is summarized in Fig 23. The media composition optimized for shoot regeneration are mentioned in **Table 7**.

Table 7. Composition of media used for regeneration of shoots from cotyledon of *B. juncea* var. PJK

Mediu m	Salts	Sucrose (%)	Agar (%)	PH	BAP (mg/l)	AgNO ₃ (20 µM)
SGM	½ MS	-	8	5.8	-	-
SRM	MS	3	8	5.8	2.5	20
SEM	MS	3	8	5.8	0.5	-
RIM	MS	3	8	5.8	-	-

SGM- Seed germination medium, SRM- Shoot regeneration medium, SEM- Shoot elongation medium, RIM- Root induction medium

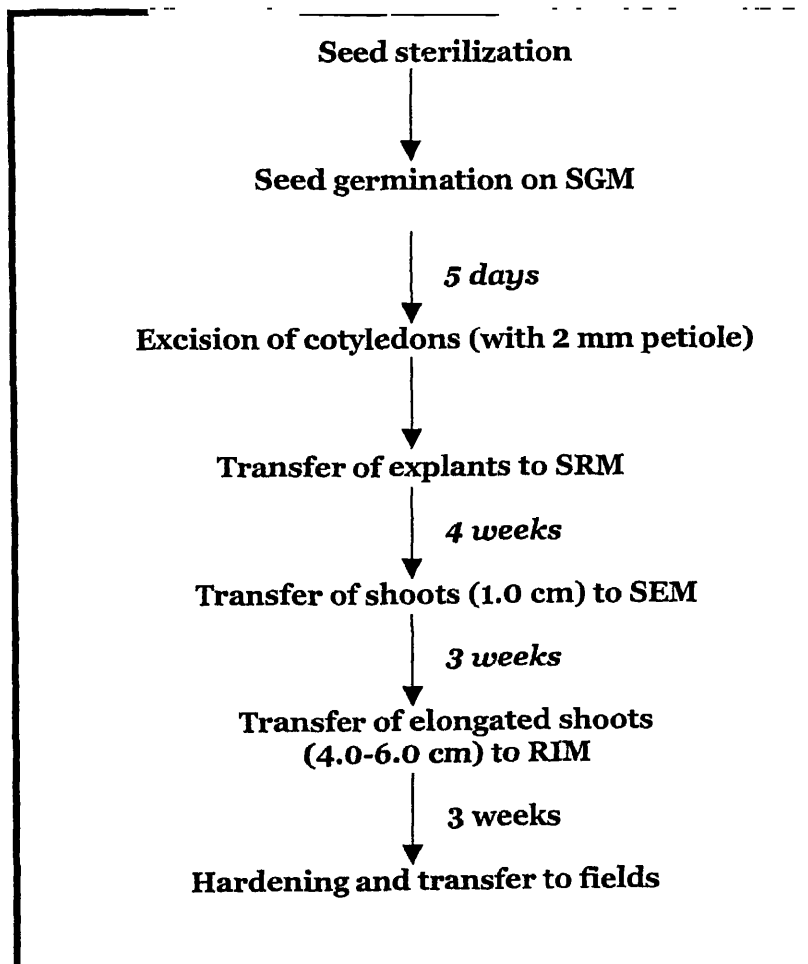


Fig. 23 Protocol optimized for shoot regeneration from cotyledonary petioles of *B. juncea* var. PJK

Genetic transformation

Protocol was standardized for the genetic transformation of cotyledonary petioles of *B. juncea* var. PJK.

Bacterial strains and constructs

Plasmid constructs used in the study were

- 1 pBI121+lec25+LAB4404
2. pBI121+lec25+EHA105
- 3 pE1226+lec25+LBA4404
- 4 pE1226+lec25+EHA105
- 5 pE1774+lec25+LBA4404
- 6 pE1774+lec25+ EHA105
- 7 pE1780+lec25+LBA4404
8. pE1780+ lec25+ EHA105

These constructs were maintained in *E. coli* strains DH- 5 α and JM 109. Plasmids were essentially isolated by the Rapid Isolation Method (Birnboim 1983).

Plasmids were mobilized into *Agrobacterium tumefaciens* strain LBA4404 by Freeze-Thaw Method (Holsters *et al.* 1978). *Agrobacterium* was grown in YEB medium at 28 °C, overnight. To the 2ml of overnight grown culture, 50 ml YEB medium was added. Cells were grown at 28 °C till an OD of 0.5- to 1.0 was attained. Cells were made competent by chilling the culture in ice and pelleting the cells by centrifugation at 4000 rpm and 4 °C for 5 min. Cells were suspended in 1 ml of 20 mM CaCl₂ solution (ice- cold). Plasmid DNA was mobilized into the cells by adding 1 μ g of plasmid DNA to 0.1 ml of the cell suspension. The cells were frozen in liquid nitrogen and subsequently thawed at 37 °C for 5 min. To the cell suspension, 1ml of YEB medium was added and the cells were grown for 2-4 hrs. Cell suspension was centrifuged for 30 s and the pellet was resuspended in 0.1 ml of YEB medium. Cells were spread in YEB plates supplemented with kanamycin (50 mg/l) and rifampicin (50 mg/l). Colonies were checked for transformation after 2-3 days.

Agrobacterium strains carrying the plasmid construct were preserved in glycerol stocks (25%) at -70 °C. They were also streaked on YEB plates.

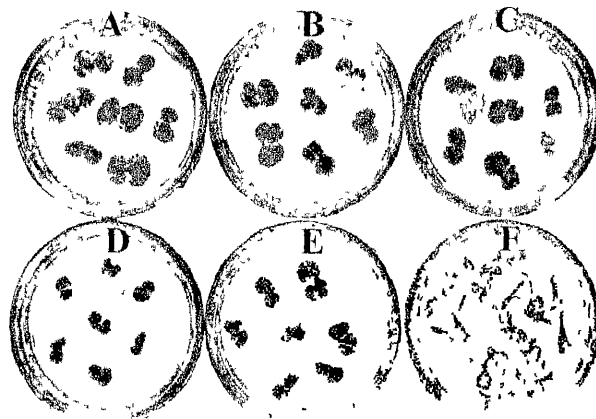


Fig. 24. Bleaching and regeneration potential of cotyledonary petiole at hygromycin concentrations of (A)-5, (B)-10, (C)-15, (E)-20, (D)-25mg/l. Positive control (F) lacks hygromycin.

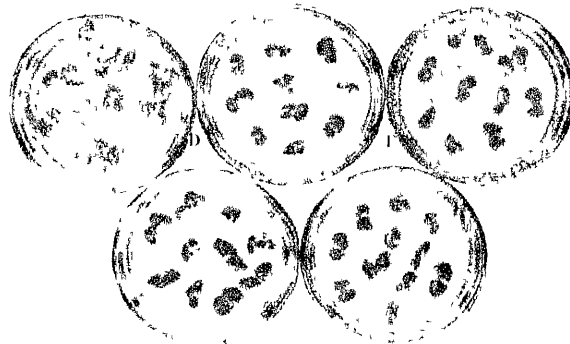


Fig. 25. Bleaching and regeneration potential of cotyledonary petioles at kanamycin concentrations of (B)-25, (C)-50, (D)-100 and, (E) 150mg/l. Positive control (A) lacks antibiotics.

Determination of natural tolerance level of explants to kanamycin and hygromycin

Inefficient selection is a major cause of poor transformation frequencies in *Brassica* species, and results in regeneration of chimeric plants upon *Agrobacterium tumefaciens*-mediated transformation. Phytotoxic level of antibiotics for plant selection (kanamycin and hygromycin) was therefore determined for efficient selection of transformants. A range of concentrations of kanamycin (0-150 mg/l) and hygromycin (0-25 mg/l) were evaluated with respect to their effect on shoot regeneration potential from 5-day-old cotyledonary petioles (Fig. 24 & 25). At a kanamycin concentration of 25 mg/l, shoot regeneration was completely inhibited, but bleaching of the explants was not observed even in presence of extremely high concentrations of kanamycin (150 mg/l). Further, it was found that with increase in hygromycin concentration extent of explant bleaching increased and was the explants were completely bleached at 20 mg/l of hygromycin. Shoot regeneration occurred at 5 mg/l of hygromycin, but at a concentration 10 mg/l it was completely inhibited. However, in both cases the control plants regenerated with high frequency.

Plant transformation

Genetic transformation of *B. juncea* var. PJK was carried out using the protocol summarized in Fig. 26. Seeds were surface sterilized with 0.1% mercuric chloride and germinated on half strength MS medium lacking sucrose. Cotyledons (with 2 mm petiole) were excised and placed on MS medium supplemented with 1 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) for a period of two days. Pre-cultured explants were infected with *Agrobacterium tumefaciens* strain LBA4404 (harbouring the plasmids pBI121+lec25+LAB4404, pBI121+lec25+EHA105; pE1226+lec25+LBA4404; pE1226+lec25+EHA105, pE1774+lec25+LBA4404; pE1774+lec25+ EHA105; pE1780+lec25+LBA4404; and pE1780+ lec25+ EHA105), for 30 min. Excess of the bacteria was blotted on to sterile filter paper and co-cultivated with *Agrobacterium* on MS medium supplemented with acetosyringone (100 μ M) for 2 days, in dark, at 22 °C (Fig 15). Subsequently, the explants were washed thoroughly with sterile water, blot dried on sterile filter paper and transferred to petridishes containing 2.5 mg/l of 6-benzylaminopurine (BAP), AgNO₃ (20 μ M) and cefotaxime (500 mg/l) for two days. Cefotaxime was added to kill the *Agrobacterium*. These explants were transferred to selection medium consisting of MS medium supplemented with BAP, AgNO₃, and cefotaxime (250 mg/l) and the appropriate concentrations of the antibiotics. Plants transformed with the binary vector pE1226 were selected on kanamycin (50 mg/l), while hygromycin (20mg/l) was used to select explants transformed with pE1774.

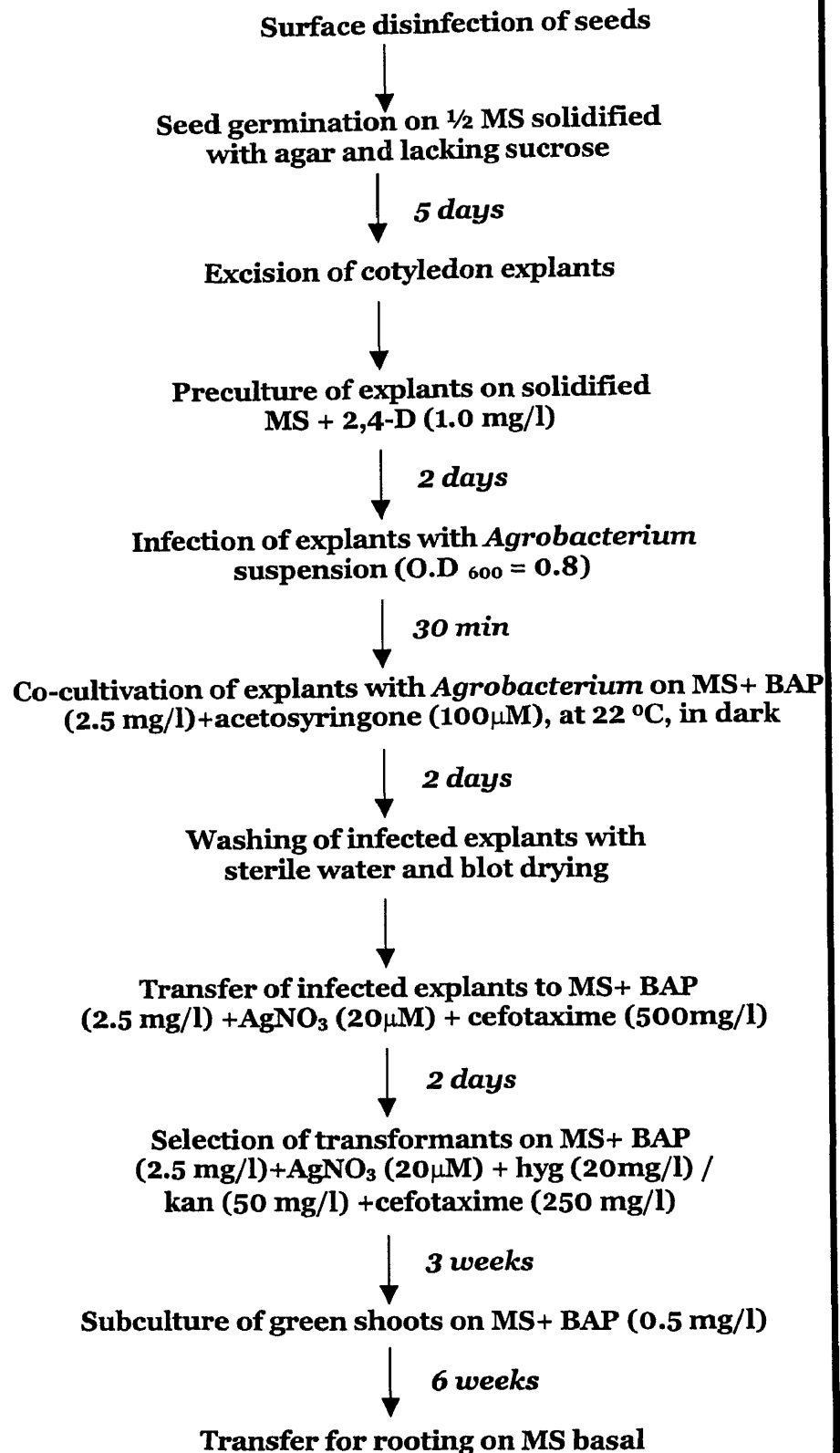


Fig 26. Strategy for *Agrobacterium* – mediated transformation of *B.juncea* var. PJK

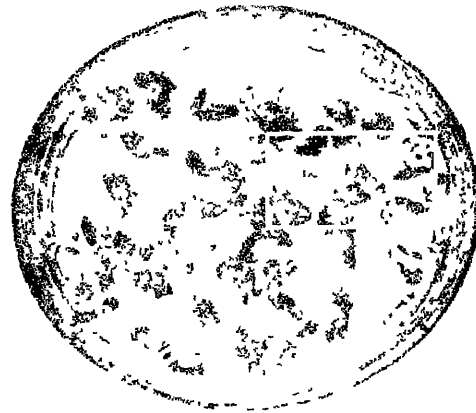


**Fig.27. Shoots from pE1226
transformed cotyledonary
petiole of *B. juncea* var.PJK**



**Fig.28. Shoots from pE1774
transformed cotyledonary
petiole of *B. juncea* var.PJK**



Fig. 29. In vitro rooting of pE1774 transformed *B. juncea* shoots on IBA supplemented medium



Fig. 30. Transformed plantlets of *B. juncea* a) during hardening; b) hardened plant in flowering.

The shoots obtained after 4 weeks of culture on selection medium were dark green in color, but stunted. These shoots were excised and transferred to shoot elongation medium devoid of antibiotics. Each green shoot was grown and multiplied as individual transgenic lines. Shoots were allowed to grow on this medium for another 3 weeks. Healthy shoots with an average height of 1.5 cm were transferred to the rooting medium.

Selection of putative transformants

More than 6500 cotyledonary petiole explants were co-cultivated with *Agrobacterium tumefaciens* strain LBA4404 carrying the plasmid pE1226 (**Fig 27**). Only 5.5% of the co-cultivated explants regenerated shoots and 3% remained green after 4 weeks of culture on medium supplemented with kanamycin.

Of the 5000 explants co-cultivated with *A. tumefaciens* strain LBA4404 harbouring the plasmid pE1774, shoot regeneration was observed only in 4.5% of the cotyledonary petioles. After 4 weeks of culture on selection medium only 2.2% of the shoots remained green (**Fig 28**). Healthy green shoots were made to root on the rooting medium (**Fig 29**). Rooted transgenic plants were initially hardened and transferred in polybags (**Fig 30a**) and finally these hardened plants were transferred in the clay pots. Transgenic plants having lectin genes showed normal growth and flowering (**Fig 30b**).

Histochemical analysis of putative transformants

More than 6500 cotyledonary petiole explants were co-cultivated with *Agrobacterium tumefaciens* strain LBA4404 carrying the plasmid pE1226. For studying the extent of transformation in early stages, 10-14 day old co-cultivated explants were checked for β -glucuronidase (GUS) enzyme activity by Jefferson's method (1987) (**Fig.31**). The explant was completely immersed in staining reagent mix which typically contains three components- the substrate, X-gluc (5-bromo-4-chloro-3-indoyl- β -glucuronide, 1.2 mM), the buffer (0.1 M sodium phosphate, pH 7.8) and the oxidation catalyst (0.5 mM each of potassium ferri- and ferrocyanide, pH 7.0, plus 10 mM EDTA, pH 7.0). Histochemical reaction was carried out at 37 °C for 6-12 hours. Tissue was cleaned with 70% ethanol and examined for blue spots.

PCR analysis of putative transformants

Total genomic DNA was isolated from fresh leaves of the *in vitro* growing putative transformants using the CTAB method of Doyle and Doyle (1987).

DNAs isolated from transformed and control plants were analyzed for the presence of *lectin* gene by PCR. Each PCR reaction was carried out in 50 µl reaction volumes containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 µM of dATP, dCTP, dTTP and dGTP, Taq polymerase (1U), genomic DNA (200 ng) and oligonucleotide primers. The oligonucleotide primers were designed for PCR amplification of *lectin* gene. Sequences of these primers are as follows.

Forward primer (F): 5'-GGTGTTCATCACACCATCTTGC-3'

Reverse primer (R): 5'-CACAAGCTTTATCTTTCCAGC-3'

The amplification reaction was carried out under following conditions – 94 °C for 4 min (one cycle), 92 °C for 60 s (denaturation), 52 °C for 45 seconds (annealing), 72 °C for 90 s (extension) for 28 cycles and final extension at 72 °C for 5 min (one cycle). The amplification products were analysed for DNA bands by agarose gel electrophoresis. To the PCR products gel loading buffer (2.5% xylene cynol, 2.5% bromophenol blue, 30% glycerol) was added and the mixture was loaded onto 1% agarose gel, prepared in 0.5X TBE (prepared on diluting 20X TBE: 0.089M Tris, 0.089 M Boric acid, 0.002 M EDTA) and containing ethidium bromide (1µg/ml). One slot was loaded with molecular weight marker (1kb ladder, Promega). The samples were subjected to electrophoresis in 0.5X TBE buffer at 60 V for 3 hours. DNA bands were visualized in UV light.

PCR analysis was carried out for the initial screening of putative transformants of *B. juncea* var. PJK transformed with pE1226, for the presence of the *lectin* gene. The DNA isolated from the shoots of the selected putative transgenic plantlets that were multiplied, was amplified using primers for *lectin* gene. About 3.5% of the putative transformants (5 plants) of *B. juncea* vars. PJK gave amplification of the 0.5 kb full length *lectin* gene (**Fig 32**). These results clearly indicate the successful integration of the gene cassette in these putative transformed lines of *B. juncea* var. PJK.

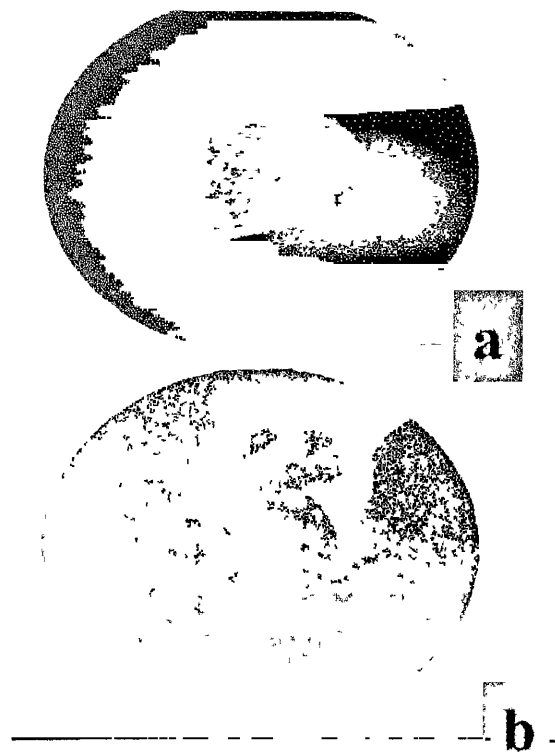


Fig. 31. Expression of GUS in: a) cotyledonary petiole explant; b) differentiated shoot form cotyledonary petiole explant of *Brassica juncea*.

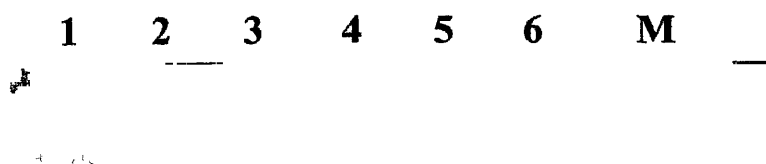


Figure 32 PCR of Transgenic plants with lectin primers. M is the marker.

References:

1. Birnboim HC (1983) A rapid alkaline extraction procedure for screening of plasmid DNA. *Methods Enzymol* 100: 243-255
2. Chopra, V L., Prakash, S., 1996. In: *Oilseed and Vegetable Brassicas: Indian Perspective*. Oxford and IBH Publishers, New Delhi, pp. 184-207.
3. De Oliveira, J. R. A., 1986. Seed lectins: the effects of dietary *Phaseolus vulgaris* on the general metabolism of monogastric animals. Ph.D. thesis. Aberdeen University
4. Devic M, Albert S, Delseny M, Roscoe TJ (1997) Efficient PCR walking on plant genomic DNA *Plant Physiol Biochem* 35(4): 331-339
5. Doyle JJ, Doyle JL (1990) Isolation of plants DNA from fresh tissue. *Focus* 12: 13-15
6. Gatehouse, A.M.R., Davison, G.M., Newell, C.A., Merryweather, A., Hamilton, W. D. O., Burgess, E P.J., Gilbert, R.J.C., Gatehouse, J.A., 1997. Transgenic potato plants with enhanced resistance to the tomato moth, *Lacanobia oleracea*: growth broom trials. *Molecular Breeding* 3. 49-63.
7. Goswami, J., Sanyal, R., Mohan, J., Dhawan, V., Lakshmikumaran, M., 1998. Genetic manipulation of crops for insect resistance In: *Plant Tissue Culture and Molecular Biology: Application and Prospects*. Narosa Publishing House, New Delhi, Madras, Bombay, Calcutta, London, pp 670-693.
8. Janzen, D.H., Juster, H.B., Liener, Ie., 1976. Insecticidal action of the phytohemagglutinin in black bean on a bruchid beetle. *Science* 192:795-796.
9. Liner, I.E., 1980. *Toxic content of Plant Foodstuffs*, 2nd edn, Academic Press, New York
10. Murashige, T., Skoog, F , 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plant.* 15, 473-497.
11. Newell, C.A., Lowe, J M., Merryweather, A., Rooke, L.M., Hamilton, W.D.O., 1995. Transformation of sweet potato (*Ipomoea batatas* L. (Lam.) with *Agrobacterium tumefaciens* and regeneration of plant expressing cowpea trypsin-inhibitor and snowdrop lectin. *Plant Science* 107:215-227.
12. Siebert PD, Chenchick A, Kellogg DE, Lukyanov KA, Lukyanov SA (1995) An improved PCR method for walking in uncloned genomic DNA. *Nucl Acids Res* 23: 1087-1088